An analysis of the immune response to primary Epstein- Barr Virus infection and the association with clinical events

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PhD – The University of Edinburgh – 2004

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I declare

(a) The work submitted for this Phd was written by Hilary Williams,

(b) The vast majority of the laboratory and clinical work was carried out by Hilary Williams. Some work was carried out on a collaborative basis and it is clearly stated in the text when this has occurred, and who the other researchers were.

(c) The work has not been submitted for any other degree or professional qualification

Hilary Williams 18/01/05

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Preface

Acknowledgements

I would like to thank everyone who helped me to complete this piece of work. One of the most satisfying aspects of submitting this PhD thesis, is the completion of a piece of work which was enabled by the generous collaboration of a large number of people. Karen Macauley, Alison Coffey (at the Sanger centre), Francis Rae, Cathy Simpson, Gwen Wilkie, Elaine Edgar, and Jill Douglas, gave me excellent and vital technical and secretarial assistance. The staff and students at the University Health Centre, who generously gave both their time and numerous blood samples; as did the staff and patients at the Regional Unit for Infectious Disease in Edinburgh. Much of the clinical work was carried out by Karen Macsween who collected the blood samples and collated the clinical information. Craig Higgens and Anthony Swerdlow both provided invaluable advise on study design and statistical analysis. Tanzina Haque, and many others provided time to discuss ideas and projects. Finally, I would like to thank Dorothy Crawford, who is an superbly stimulating and exemplary supervisor.

This work was funded by the Medical Research Council of Great Britain; both by a Clinical Training Fellowship and Project Grant to Dorothy Crawford.

In addition I would like to thank Richard and my parents for providing me with the time and energy to complete this project, and Jill for looking after Natasha so well.

Abbreviations and symbols

AIDS	Acquired immunodeficiency syndrome
BL	Burtkitt Lymphoma
BLPD	B cell lymphoproliferative disease
BME	b- mercaptoethanol
Bp	Base pair
BSA	Bovine serum albumin
CD	Cluster designation
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CyC	Cy-Chrome™
DAB	3, 3'- diaminobenzidine
DNA	Deoxyribonucleic acid
DNTP	2'deoxynucleoside 5'-triphosphates
Ds	Double stranded
EA	Early antigen
EBER	EBV-encoded small RNA
EBNA	EBV nuclear antigen
EBV	Epstein Barr Virus
EDTA	Ethylene diamine terta-acetic acid
ELISA	Enzyme-linked immunoabsorbant assay
EtBr	Ethidium Bromide
FACS	Flourescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein-5-isothiocyanate
HCL	Hydrochloric acid
HD	Hodgkin's disease
HHV	Human herpes virus
HIV	Human immunodefiency virus
IHC	Immunohistochemistry
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin

IM	Infectious mononucleosis
HBSS	Hanks balanced salt solution
HIV	Human immunodeficiency virus
L	Litre
LCL	Lymphoblastoid cell line
MACS	Magnetic affinity cell separation
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MHV-68	Murine gamma herpes virus 68
NCBI	National Center for Biotechnology
NHL	Non Hodgkin's lymphoma
NK cell	Natural killer cell
NPC	Nasopharyngeal carcinoma
OD	Optical density
PBS	Phosphate buffered saline
PBMC/PBM	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PE	R-phycoerythrin
PHA	Phytohemagglutinin
PMA	Phorbal 12-myristate 13-acetate
Rpm	Revolutions per minute
SCID	Severe combined immunodeficient
T.O.1.E	Tris HCL EDTA
TCR	Tris HCL EDTA with creol red
Topro-3	Topro –3 iodide
TPBS	TRIS PBS
TCR	T cell receptor
TNF	Tumour necrosis factor
VCA	Virus capsid antigen
V/V	Volume per volume
VZV	Varicella zoster virus
XLPS	X linked lymphoproliferative syndrome
XLP	X linked lymphoproliferative disease

u	Micro
α	Alpha
β	Beta
χ	Gamma

Abstract

Infectious Mononucleosis (IM) is one of the most common serious illnesses in young adults, and is caused by delayed primary infection with Epstein-Barr Virus (EBV). The characteristic clinical features are thought to be due to extensive T cell activation, and cytokine production, but the molecular mechanisms underlying this are unclear. SAP (Surface Lymphocyte Activation Marker (SLAM) associated protein), is mutated in X-linked lymphoproliferative disease (XLP), in which fatal IM occurs, and is a key regulator of lymphocyte activation via signals from cell surface 2B4 (CD244) and SLAM. Our aim was to monitor T cell activation via this SAP/SLAM/CD244 pathway, and analyse whether the scale of activation was related to the severity of clinical features in a cohort of 26 cases of IM. In addition, 10 males with fulminant IM were screened for XLP gene mutations but none were identified.

At diagnosis of IM, SAP, CD244 and SLAM were significantly upregulated, compared to controls, on both CD4 and CD8 T cells in peripheral blood ($p<0.01$). The expression fell over the course of IM, but CD244 and SLAM remained elevated on CD8 cells at one month post diagnosis. The numbers of lymphocytes expressing CD8, and CD244/CD8 were significantly higher in cases with severe compared to mild sore throat ($p<0.05$). Both expression of CD8, and expression of CD244 on CD8 cells correlated positively with increased viral load ($p<0.05$).

Time is required for this antigen specific immune response to develop. In contrast innate immune responses, such as Natural Killer (NK) cells are thought to be vital early in the infection process. We show that NK cell numbers are significantly elevated at diagnosis of IM compared to controls ($p<0.01$) and in the first month following diagnosis.

In healthy adults, 2 distinct populations of NK cells have been identified by the density of cell surface expression of CD56; these subsets of $CD56^{bright}$ and $CD56^{dim}$ cells differ in their ability to produce cytokines and lyse target cells. We have identified significant changes in NK cell phenotype and function during IM, with an increase in the proportion of $CD56^{bright}$ cells, and cells showing an enhanced ability to kill an EBV infected cell line.

We suggest that activated T cells expressing CD244 modulate the clinical features of IM, but control of activation is maintained by concurrent increased expression of SAP. However, before this occurs NK cells have a critical role in both eliminating infected B cells and augmenting this antigen specific T cell response via release of immunomodulatory cytokines. The magnitude of the NK cell response may ultimately determine whether primary EBV infection has a subclinical or clinical outcome.

1 Aims

- To screen a series of 10 males with fulminant IM or unexplained severe primary EBV infection for mutations in the XLP gene SAP/SH2D1A.

- To establish a cohort of young adults with IM, and to collect concurrent data on the clinical course of the illness from diagnosis to recovery, and samples for laboratory analysis (serum, viable PBMC, and DNA).

- To investigate the expression of the SAP/SLAM/CD244 lymphocyte activation pathway, which is mutated in XLP, at diagnosis and during recovery from IM in healthy young adults. To analyse the relationship between the scale of this immune response and the clinical features of IM in this cohort.

- To investigate the scale, phenotype, and function of the innate natural killer cell response at diagnosis and during recovery from acute IM.

2 Introduction

2.1 Herpes Viruses

Epstein Barr Virus (EBV) is a member of the herpes virus family; all herpes viruses have a comparable structure, and typically this consists of a core of linear double stranded DNA, surrounded by a protein tegument and an envelope with viral glycoprotein spikes (Roizman B, 1996).

All herpes viruses have a similar life cycle exemplified by their ability to persist in the host for life. In the vast majority of healthy carriers the viruses cause limited or no disease. This is because a delicate balance is maintained between the host immune system, which limits production of new virus, and the virus, which persists and is successfully transmitted in the face of host anti viral immunity. Disruption of this balance, due to primary or acquired abnormalities of the host's immune system, may lead to the development of virus associated disease.

Three subfamilies of herpes viruses are recognised, alpha, beta and gamma, and this division has been made on the basis of biological properties, such as host range and site of latency (Roizman B, 1996). Eight different herpes viruses are recognised to infect humans: these are herpes simplex virus (HSV) 1 and 2, varicella zoster virus (VZV), human cytomegalovirus (HCMV), human herpes virus 6 and 7 (HHV6 and 7), EBV, and Kaposi's sarcoma associated herpes virus (KSHV). The clinical conditions associated with both primary infection and reactivation are detailed in Table 2-1 (page 18), additionally the subfamily to which the virus belongs is shown. The chief characteristics of the gamma herpes viruses, of which EBV is a member, are target cells of lymphoid and in some cases epithelial cell origin, and a site of latency which is frequently lymphoid tissue.

The EBV genome was the first herpes virus to be sequenced; it consists of a linear, double stranded 184-kilobase pair DNA (http://www.med.ic.ac.uk/ludwig/EBV.htm). EBV has coding potential for about 70 proteins. The genomes of the herpes viruses vary from 31-75 percent guanine or cytosine, and that of EBV is composed of 60 percent guanine or cytosine (Pritchett et al., 1975).

The other herpes virus discussed in this thesis is VZV, which has a genome of 125000 base pairs (Davison and Scott, 1986). The early genes induce replication of viral DNA, and include viral thymidine kinase, a key target of anti viral drugs. The late genes encode structural components of the virion, the viral glyoproteins and envelope. The viral glycoproteins, VZV gH and VZV gC are both thought to mediate viral entry and are targets of neutralising antibody.

Table 2-1 Human herpes virus infections and associated diseases.

Virus	Subfamily	Disease Association	
		Primary infection	Reactivation
Herpes simplex virus 1	Alpha	Cold sore	Cold sore
Herpes simplex virus 2	Alpha	Genital herpes	Genital herpes
Varicella zoster virus	Alpha	Chicken pox (varicella)	Shingles (Zoster)
Cytomegalovirus	Beta	IM – like illness	Retinitis, Colitis, Pneumonitis
Human herpes virus 6	Beta	Exanthum subitum (Roseola infantum)	Unknown
Human herpes virus 7	Beta	Exanthum subitum (rarely)	Unknown
Epstein Barr virus	Gamma	Infectious mononucleosis	Oral hair leukoplakia Epithelial and lymphoid tumours
Kaposi sarcoma herpes virus	Gamma	Unknown	Kaposi sarcoma Castleman's Disease Primary effusion lymphoma

2.2 Epstein-Barr Virus

EBV is one of the most studied herpes viruses and has particularly attracted interest because of its oncogenic potential. Indeed, EBV was first identified in cultures from biopsies of Burkitt's lymphomas from Africa (Epstein MA, 1964); and shortly

afterwards was reported to transform B cells to long lived lymphoblastoid cell lines (Pope et al., 1968a).

The link between EBV and infectious mononucleosis (IM) was also quickly established. EBV was identified as the culprit virus when a female technician working in a laboratory studying EBV developed IM, and was found to have developed EBV antibodies during the course of the acute illness (Henle et al., 1968). Subsequently, a serological study was undertaken of new students at Yale University, and thus delayed primary EBV infection was confirmed to be the most common aetiology underlying acute IM (Niederman et al., 1970). These studies are discussed in more detail later in the section on IM (Section 2.6.1 page 28). Over the next ten years it also became apparent that very occasionally primary EBV infection could cause a severe, often fatal illness, known as fulminant IM (Purtilo et al., 1975). The recognition that this occurred in maternally related male children led to the identification of the X linked lymphoproliferative syndrome (XLPS).

Interest in EBV has continued over the subsequent 35 years with the recognition that the virus plays a central role in the development of a number of lymphoid and epithelial cell-derived cancers. Many EBV linked tumours show marked geographical variation. China and South East Asia have the highest prevalence; where both the majority of nasopharngeal carcinomas (Wolf et al., 1973; zur et al., 1970) and up to 15 percent of stomach cancers are linked to EBV (Shibata and Weiss, 1992). EBV is linked with childhood Burkitt's lymphoma in Africa (Epstein MA, 1964) and throughout the world is recognised to have an important role in the aetiology of Hodgkin's disease (Weiss et al., 1989). Other groups of tumours occur in the immunosuppressed, and these include AIDS related non-Hodgkin's lymphoma (NHL) (Boshoff and Weiss, 2002), and B cell lymphoproliferative disease (BLPD) (Crawford et al., 1980). EBV related tumours are not discussed any further, however. Table 2-2 (page 20) and Table 2-3 (page 21) provide a summary of EBV associated diseases.

Table 2-2 EBV associated diseases of lymphocyte origin

Disease	Population at risk	EBV association
Infectious Mononucleosis	Young adults in the West	Majority
X-linked lymphoproliferative disease (XLP)	Males with mutations in the XLP gene	Majority
B lymphoproliferative disease (BLPD)	Post transplant LP HIV infection- primary central nervous system lymphoma & peripheral lymphoma	>90% 100%
Burkitt's lymphoma (BL)	African children HIV infection	97-100% >25%
Hodgkin's disease (HD)	Children –developing countries Young adults- high socioeconomic groups and history of IM	40-80% depending on type
T/NK lymphoma	Chronic active EBV HIV infection	10-100% depending on type
Primary effusion lymphoma	HIV infection	70-80%. 100% contain KSHV DNA.

Table 2-3 EBV associated disease of epithelial cell origin

Disease	Population at risk	EBV association
Oral hairy leukokplakia	HIV and other immunodeficiencies	100%
Nasopharyngeal carcinoma	S.Chinese and Inuit races- high incidence Mayaks, Dyaks, Indonesians, Filipinos, Vietnamese- moderate incidence	Undifferentiated carcinoma 100%
Gastric carcinoma	Not identified	Adenocarcinoma 5-15%
Leiomyosarcoma	HIV and immunodeficiency	Not known

2.3 Epidemiology of EBV

Primary infection with EBV is commonly sub-clinical, occurs predominately in early childhood, and gives rise to a lifelong carrier state (Fleisher et al., 1979). In most populations seropositivity increases with age, and over 90% of adults have persistent infection. However, the rate of seroconversion varies according to economic status. Over 90% of children over 2 years in developing countries have evidence of persistent infection (Biggar et al., 1978; Haque et al., 1996), whereas seroconversion may be delayed until adolescence in high socio-economic groups in industrialised countries (2.6.1 page 28). The virus is present in saliva (Gerber et al., 1972), and it is thought that close family contact is the main route of spread in young infants, and therefore large family size and close proximity of living conditions are likely to aid spread. In the west 5-10 % of people remain EBV negative for life, whereas in Africa over 97% of the population carries the virus (Essers et al., 1991). The reasons for this difference remain unclear.

2.4 Virus subtypes

Most healthy hosts carry only one of the two major virus subtypes of EBV, 1 or 2 (also called A and B respectively). The subtypes have 70-80% sequence homology, and differ mainly in the genes which encode EBV nuclear antigens (EBNA)-2, -3A, -3B and -3C (Rickinson and Kieff, 2003). Type 1 is the most common throughout the

world; type 2 is rare, but is more prevalent in Africa and the Far East than the West (Zimber et al., 1986). In addition to the subtypes, minor sequence variants are classified as strains and are usually unique to one individual, although recent data suggest even healthy individuals may harbour more than one strain (Sitki-Green et al., 2003). There is no evidence that variation of either strain or type affects clinical pathogenesis.

2.5 EBV life cycle and patterns of viral latency

2.5.1 Cellular events of primary infection

EBV infects B cells via binding of the major viral envelope glycoprotein (gp 350) (Tanner et al., 1987) to the CD21 receptor on resting B cells (Nemerow et al., 1986). There has been a long standing debate as to additional cell types targeted in healthy carriers; viral colonisation of B cells is well defined but whether infection also occurs in epithelial cells is still under debate (Faulkner et al., 2000). Several histological studies of IM tonsils have identified infected B cells around the tonsillar crypts, but did not find any evidence of infection of epithelial cells (Niedobitek et al., 1989). Additionally, a study on individuals with X-linked agammaglobulinaemia, who lack B cells, found no evidence of either past or present EBV infection. This suggests EBV colonisation can not occur in the absence of normal B cells (Faulkner et al., 1999).

On the other hand EBV can be detected in and definitively linked to the pathogenesis of epithelial cell diseases. These include oral hairy leukoplakia (Greenspan et al., 1985), a benign infection of squamous epithelial cells on the tongue that occurs in the immunosuppressed, and malignant nasopharyngeal carcinoma (Raab-Traub, 1992). One possible explanation for the difficulties in identifying EBV infection in epithelial cells from healthy virus carriers is that the infection is a transient event. Borza and Hutt-Fletcher identified differences between the viral progeny of epithelial and B cells in vitro, and showed that these changes identified in the viral envelope were associated with changes in viral tropism. They suggest that epithelial cells are transiently infected in primary infection, but that virus produced at this site is

particularly effective at infecting B cells, and thus establishing viral persistence (Borza and Hutt-Fletcher, 2002).

2.5.2 Three stages of viral life cycle

EBV, like all herpes viruses, establishes lifelong infection within the human host. One of the particularly interesting features of EBV is that the site of viral persistence, the B cell, is a key component of the host immune system. Recently progress has been made in understanding how the virus exploits physiological pathways of B cell differentiation in order to maintain this life long infection (Thorley-Lawson, 2001). Three different stages of viral life cycle can be recognised. The first is viral entry into and induction of proliferation of the host B cell. The second is viral persistence, when the virus remains latent in B cells (viral latency). The third stage is viral reactivation, and production of viral progeny to infect both new cells within the host and allow transmission to naïve hosts (lytic infection). (Bornkamm and Hammerschmidt, 2001) have emphasised the importance of understanding that these three processes occur simultaneously within the healthy seropostive host . Historically, our understanding of the molecular events underlying gene expression during the viral life cycle has been from in vitro studies.

2.5.3 Patterns of viral latency

The ability of EBV to induce B cell proliferation, in vitro, was established over 35 years ago; following infection, the resting B cell is transformed into an activated phenotype capable of long term growth, known as a lymphoblastoid cell line (LCL) (Henle et al., 1967; Pope et al., 1968b). Studies of the genetic events underlying B cell transformation have aided our understanding of the processes underlying viral persistence in vivo and the role of EBV in tumour pathogenesis.

Table 2-4 EBV latent antigen expression

EBV antigen	Function known/postulated	Expressed in vivo in							
		PBM	IM	BL	NPC	HD	BLPD	TCL	Ga Ca
EBNA 1	Genome maintenance	?	+	+	+	+	+	+	+
EBNA 2	Viral oncogene, transactivates cellular and other viral genes	-	+	-	-	-	+	-	-
EBNA 3A	Activates cellular genes	-	+	-	-	-	+	-	-
EBNA 3B	Activates cellular genes	-	+	-	-	-	+	-	-
EBNA 3C	Viral oncogene, increases expression of LMP1	-	+	-	-	-	+	-	-
EBNA LP	Co-activates EBNA –2 responsive genes, increases efficiency of immortalisation	-	+	-	-	-	+	-	-
LMP 1	Viral oncogene, induces B cell activation and adhesion, inhibits apoptosis	-	+	-	(+)	+	+	(+)	?
LMP 2	Repression of lytic cycle, enhances B cell survival	+	+	-	+	+	+	+	+

PBM- peripheral blood mononuclear cells, **IM**-infectious mononucleosis, **BL-** Burkitt's lymphoma, **NPC**- nasopharngeal carcinoma, **HD**-Hodgkin's Disease, **BLPD-** B cell lymphoproliferative disease, **TCL-** T cell lymphoma, **Ga Ca**- gastric lymphoma. + - expressed, - not expressed

Three dominant patterns of latent gene expression are recognised from these in vitro studies, and are known as latency 1, 2 and 3 (Rickinson and Kieff, 2003). Latency 3 is also known as the growth program (Thorley-Lawson, 2001). In latency 3, all 9 latent genes are expressed and these are EBNA –1, -2, -3A,- 3B, -3C, and - LP(leader protein), Latent membrane proteins (LMP) 1, 2A, and 2B, and two small nonpolyadenylated RNAs (EBERs) (Dorothy H Crawford, 2001). EBNA –3A, -3B, and 3C are also known as EBNA 3, 4 and 6, respectively. In addition in latency 3, the BamHI A RNAs are transcribed, but little is known about their function (Epsten MA Crawford DH, 1996). In latency patterns 1 and 2, a much more limited pattern of

gene expression occurs. This classification of viral latency is useful, but it should be recognised that some overlap between the patterns of gene expression occurs in vivo. Table 2-4 (page 24) summaries the pattern of gene expression that occurs in EBV linked conditions of both lymphocyte and epithelial cell origin.

2.5.4 Virus induced proliferation of B cells

Following EBV infection of a naive B cell, all 9 latent viral genes are expressed, (latency 3) and these induce the B cell to enter cycle and proliferate (Bornkamm and Hammerschmidt, 2001). The first gene to be transcribed is EBNA–2 (Allday et al., 1989; Henkel et al., 1994; Rooney et al., 1989), which in co-ordination with EBNA LP (Harada and Kieff, 1997), provides critical transactivation of both the cellular and viral genes required for this process to occur. EBNA-3A and EBNA-3C are all required for initiation of transformation and subsequent activation of cellular genes. LMP-1 is essential for the proliferation of B cells (Peng and Lundgren, 1992). EBNA -1 is able to attach to cellular chromosomes and so ensures the maintenance of EBV in daughter cells (Yates et al., 1984).

LMP-1, EBNA-2 and EBNA- 3C are all recognised as viral oncogenes (Bornkamm and Hammerschmidt, 2001). LMP-1 can transform rodent fibroblasts, and induce tumours in transgenic mice (Wang et al., 1985), and is widely expressed in EBV linked tumours such as Hodgkin's lymphoma (Pallesen et al., 1991) and NPC (Chen et al., 1992; Liebowitz, 1994; Niedobitek et al., 1992). EBNA-2 can regulate the cellular c-myc gene (Kaiser et al., 1999) and also viral LMP-1 (Abbot et al., 1990). EBNA-3C also regulates LMP-1(Allday et al., 1993), and can functionally inactivate the retinoblastoma gene (Cannell et al., 1996), a cellular tumour suppressor gene.

The two small non-polyadenylated RNAs (EBERs) are the most highly expressed RNAs in latently infected cells (Howe and Steitz, 1986). The demonstration of EBERs expression by in-situ hybridisation on histological sections is often used as a marker of EBV infection of a cell. However, their function is less clear although recent data suggest they may also have a role in oncogenic transformation, via the upregulation of Bcl-2 (Nanbo and Takada, 2002).

2.5.5 Viral persistence

The second stage of the viral life cycle is viral persistence and the resting memory B cell is thought to be the major site of viral persistence (Miyashita et al., 1995). In healthy seropositive adults, 0.5-50 cells per million B cells in the peripheral blood are infected with EBV (Wagner et al., 1992). Thorley-Lawson has suggested that in order to enter a state of viral persistence, changes in viral gene expression must occur which allows the B cell phenotype to change from a proliferating B cell blast into long lived memory cells (Thorley-Lawson, 2001). EBV is thought to be able to mimic, via changes in latent gene expression, the physiological signals that occur after B cell activation via antigen, and which normally induce this change in B cell phenotype.

Normally antigen activated B cells migrate to lymph nodes and proliferate in the germinal centre. Depending on the specificity of the antibody produced and its ability to bind antigen, the cell will either die by apoptosis, or survive as a resting B memory or plasma cell. The signals that allow transition to a resting cell phenotype and avoidance of apoptosis are ligation of CD40 and signalling via the B cell receptor. The latent viral genes LMP1 and LMP2A are thought to substitute for these signals in an EBV infected B cell and thus allow the cell to differentiate to a long lived B cell with latent EBV infection (Gires et al., 1997). B cells found in the peripheral blood of healthy carriers express very limited numbers of viral genes. Normally only EBERs (Tierney et al., 1994) and LMP2A (Qu and Rowe, 1992) can be detected; LMP2A prolongs cell survival by suppression of viral reactivation and entrance to the lytic cycle (Miller et al., 1994; Miller et al., 1995). For the B cell to exit the proliferation stage EBNA 2 must be switched off, and it is not clear what signals this change in gene expression.

The limited number of genes expressed in circulating B cells is thought to serve two ends towards maintaining viral persistence. Firstly the growth promoting genes are switched off, and so reducing the chance of oncogenic transformation. Secondly, minimal viral antigens are exposed to the immune system so avoiding recognition by cytotoxic lymphocytes (CTL) (Bornkamm and Hammerschmidt, 2001). In addition,

latently infected B cells do not express the co-stimulatory molecules expressed by proliferating cells, such as CD21 and CD23, which promote immune activation.

2.5.6 Viral reactivation and entry to the lytic cycle

The third stage of the viral life cycle is reactivation and entry into the lytic cycle with production of viral progeny. Expression of the BZLF1 gene is associated with the switch from latent to lytic cycle (Countryman and Miller, 1985), however it is not clear what signals this change in gene expression.

The EBV lytic cycle genes, like other herpes viruses, are described as immediate early, early and late genes. The immediate early and early genes are expressed before viral DNA is synthesised and the late genes after DNA synthesis. The late genes products include gp350/220, the major virus envelope glycoprotein (Thorley-Lawson and Poodry, 1982), which binds to CD21 (Tanner et al., 1987) and is an important target of antibody mediated immunity.

In a proportion of healthy carriers virus is continually shed in saliva allowing transmission to a new host and over time replacement of the pool of EBV infected B cells in an individual. Lytic virus has been detected in the tonsil (Ikeda et al., 2000), and it appears that circulating memory cells may return to the tonsil on reactivation and thus allow these processes to occur.

Thus in the normal host the virus maintains a dynamic equilibrium between persistent and lytic infection, and continually replenishes the pool of infected B cells.

2.6 Infectious Mononucleosis

IM is one of the most common causes of prolonged illness in university students in the West, with 5-10% affected annually (Niederman et al., 1970; Sawyer et al., 1971; Anonymous, 1971; Hallee et al., 1974), IM is most frequently caused by delayed primary infection with EBV, although delayed CMV results in similar clinical features, but only EBV infection will be discussed. Typical clinical features of IM include fever, lymphadenopathy, sore throat and prolonged fatigue. Full recovery in 6-8 weeks is the norm; however the severity and duration of symptoms are highly variable, with rare but well recognised chronic and fatal outcomes (Cohen, 2000).

In this section the epidemiology and clinical features of IM are discussed. This is followed by the immune response to both primary and persistent infection, as much of this work stems from studies of acute IM (see 2.6.8 page 34). This is followed by a review of XLP and associated syndromes in which severe primary EBV occurs (See 2.7 page 39).

2.6.1 Seroepidemiology of IM

In the late 1960's, shortly after identification of the link between primary EBV infection and IM, at least five serological studies were undertaken of both young army recruits and university students in the USA and UK (Niederman et al., 1970; Sawyer et al., 1971; Anonymous, 1971; Hallee et al., 1974; Brodsky and Heath, Jr., 1972).

Table 2-5 Summary of seroepidemiological studies of IM

	Number recruited	% positive at entry	% of EBV seronegative cases who seroconverted and developed IM
Yale University Freshmen (Niederman 1970)	424	26%	44%
Yale University Freshmen (Sawyer 1971)	355	51%	At least 74%
Students entering English Universities (P.H.L.S 1971)	1457	57%	50%
USA Military Academy (Hallee 1974)	1401	63.5%	26-35%
Range		26-63.5%	26-74%

All groups measured EBV antibodies in serum on arrival at university (or army academy) and at a time point between 9 months and 4 years later. Notably, only the UK study included female cases. In addition, Brodsky and Heath collated data from the American College Health Association and found an incidence of IM of around 10%, and this study included both male and female students (Brodsky and Heath, Jr., 1972). Thus the studies provided useful data on EBV seroprevalence in young adults, and their subsequent risk of IM (See Table 2-5 page 28).

In addition, the studies investigated the link between an individual's social and geographical status, and EBV seroprevalence. As well as studying US university students, Niederman et al measured prevalence of antibodies in other groups of young adults of comparative age with a range of social backgrounds. They found markedly higher rates of antibody positivity (75-77%) in students at universities in the Philippines, and also in military recruits in Columbia (87%), than in the US institutions (Niederman et al., 1970). Hallee et al found that military cadets from low income families, had significantly higher EBV antibody prevalence at arrival at the academy compared to those from high income families (Hallee et al., 1974).

In summary, the results confirmed that EBV seroconversion is linked to IM, and identified those at high-risk for IM as affluent groups in the West, especially new university students. The range of those who developed symptomatic seroconversion was wide, from 26-74%, and the reasons behind this are unclear. Interestingly the study in the US military academy noted a peak incidence of IM in February, about 6 weeks after the cadets Christmas leave presumably because the cadets were more likely to be sexually active while on holiday (Hallee et al., 1974).

By their mid twenties the majority of adults will be seropositive, although some, around 10%, will remain seronegative throughout adult life (Henle and Henle, 1980).

2.6.2 Transmission of EBV

IM is colloquially known as the 'Kissing Disease', and it is presumed that the initiation of sexual activity in adolescence places seronegative individuals at risk of contact with infectious virus in saliva (Gerber et al., 1972). In fact kissing was suggested as a mode of transmission even before isolation of EBV (Hoagland, 1975). Infectious virus is present in genital secretions of both male and female asymptomatic carriers (Sixbey et al., 1986; Israele et al., 1991), as well as in the saliva, so primary infection may occur when the virus is transferred to naïve cells either in the oropharynx or genital tract of the new host.

Current data, although limited, suggest that EBV is more commonly found in saliva compared to sexual secretions. Around 50% of healthy seropositives secrete EBV in their saliva (Haque and Crawford, 1997), whereas around 10% of seropositive

women who were found to have EBV present in cervical secretions (Enbom et al., 2001).

2.6.3 The incubation period of IM

At present most authorities describe a time lag of between 30–50 days after exposure to the virus before the onset of clinical IM. This time scale probably stems from the original studies of Hoagland who suggested that IM was contracted from kissing another person with IM (Hoagland, 1975). Having developed this hypothesis, he then questioned cases about their sexual activity, and calculated an incubation period of 33-49 days. We now know that his theory was partially correct, but also that primary EBV infection can be acquired from asymptomatic carriers. Additional data on the incubation period are available from case histories in which the likely date of transmission has been documented; an incubation period of 38 days was suggested in one case (Svedmyr et al., 1984) and 90 days in the other (Chang, 1975). IM can also be transmitted through blood transfusion, and studies on this mode of transmission suggest an incubation period of 21-35 days (Gerber et al., 1969). It remains difficult to establish any more accurate data on the length of the incubation period, or why it should be so prolonged.

2.6.4 Diagnostic criteria of IM

The clinical syndrome of IM was described as early as 1940, long before primary infection with EBV was identified as the most common aetiology, and the diagnostic criteria used at this time are still useful today (Hoagland, 1975):-

1. 'Clinical. The patient should present with a history of insidious onset of malaise, fever, and sore throat, and on physical examination we should find cervical lymphadenopathy and hyperplasia of pharyngeal lymphoid tissue, usually accompanied by pharyngitis.

2. Haematological. There should be an absolute lymphocytosis (4,500/mm^3), at least 51% lymphocytes on differential count, the presence of atypical lymphocytes, and the persistence of lymphocytosis for at least two weeks.

3. Serological. Heterophil (Paul-Bunnell-Davidsohn [PBD]) antibody, absorbable by ox erythrocytes but not by guinea pig kidney powder, must appear in the serum during illness'.

Current practice would continue to rely on these characteristic clinical features for making a diagnosis, but supported by EBV serology. The gold standard for the diagnosis of IM is detection of IgM antibodies to viral capsid antigen (VCA). Anti-VCA IgM is present for 1-2 months and subsequently anti- VCA to IgG appears and then remains. Most laboratories use an ELISA as a screening method, but may supplement this with an indirect immunofluorescence assay as a more sensitive test. The test described by Hoagland, the heterophile antibody test, which forms the basis for the rapid monospot test, is still used for diagnostic screening. This is positive in 85% of acute IM cases, but may be absent in young children (Sumaya and Ench, 1985). On occasions auto antibodies such as cold agglutinins, cryoglobulins, antinuclear antibodies or rheumatoid factor arise. It should be noted that although the majority of individuals with IM have a profound lymphocytosis, with 'atypical' lymphocytes (activated CD8+ T cells) in the peripheral blood, the presence of these cells is not diagnostic of IM. Atypical lymphocytes are also found to a lesser extent in other infections, including HIV seroconversion illness, CMV, viral hepatitis, toxoplasmosis, influenzae and mumps.

EBV DNA viral load is not routinely measured in IM, and the relationship between severity of illness and EBV DNA levels is not clear at present. Several investigators have compared EBV DNA viral load in healthy seropositive controls with those found in IM, and identified both a higher rate of detection and higher values in those with IM (Berger et al., 2001; Hopwood et al., 2002). In addition, the authors of a case report have suggested it is useful to monitor viral load in those with fulminant IM, in order to assess response to treatment (van Laar et al., 2002).

2.6.5 The characteristic clinical features of IM

IM normally occurs in young adults and the majority of patients experience fever, lymphadenopathy, sore throat and fatigue. Although the data are several decades old, the clinical manifestations documented in the early studies are still pertinent. The 6

key features emphasised are listed below, followed by the frequencies of occurrence as documented by (Chang r s, 1980) as originally described in (S C Finch, 1969).

1. Onset of illness is invariably insidious.

2. Headache is generally mild except in a few patients who may have severe headache (occurs in 40-70% cases).

3. Sore throat is the most common presenting symptom; it varies from mild to severe. In some patients, it may be sufficiently severe to cause pain on swallowing (occurs in 80-85% of cases).

4. About 90% of IM patients have fever, which may last up to 6 weeks (occurs in 80-95% of cases).

5. Virtually all patients have enlarged cervical lymph nodes which vary in size from that of a pea to a hazlenut, which show no sign of acute inflammation, and which are not matted together (occurs in 100% of cases).

6. Pharyngitis varies greatly in severity from mild pharyngeal injection to follicular tonsillitis or exudative pharyngitis (occurs in 65-85% of cases).

In addition, generalised lymphadenopathy often occurs accompanied by splenomegaly. A degree of hepatomegaly and mildly abnormal liver function tests are also common, but jaundice is rare (~5%). Fatigue occurs in 90-100% of cases (S C Finch, 1969). Urticarial skin rashes can occur, most commonly associated with the use of ampicillin. The aetiology of this is unclear, however a small investigation has suggested the rash is linked to a specific sensitisation to amoxicillin. Clinically this is important as this implies a long term allergy to amoxicillin, rather than a reaction limited to the inter-current viral illness (Renn et al., 2002). Rare complications include acute liver necrosis, splenic rupture, pharyngeal or tracheal obstruction, and haematological disorders, including thrombocytopenia and haemolytic anaemia (Table 2-6 page 33). Again, the majority of these features were described in the early studies on EBV (S C Finch, 1969). The aetiology of many of these complications is unclear, but the nature of the conditions (such as thrombocytopenia or glomerulonephritis) suggests either an auto immune or antibody mediated pathology.

Rare cases of IM have also been documented in children, middle-aged adults and the elderly (Auwaerter, 1999). The illness tends to be milder in young children, and more severe in older adults; the rarity and atypical presentation of IM in these age groups may make diagnosis more difficult.

Table 2-6 Complications of IM (Macsween and Crawford, 2003)

Organ/system	Complication
Liver	Jaundice (5%), abnormal liver function (~90%), fulminant hepatitis (rare)
Respiratory	Respiratory tract obstruction due to tonsillar enlargement, interstial pneumonitis (rare).
Neurological	Guillan Barre syndrome
Spleen	Splenomegaly and rupture
Haematological	Thrombocytopenia and haemolytic anaemia
Secondary infection	Streptococcal sore throat
Psychological	Depression and prolonged fatigue
Renal	Haematuria, interstial nephritis, glomerulonephritis
Cardiac	Myocarditis, pericarditis, arrythmia
Immunological	Depressed cell mediated immunity

2.6.6 Clinical course and treatment

In the vast majority of cases IM is a benign and self-limiting disease with prolonged fatigue being the most disabling symptom, and full recovery after 6-8 weeks is the norm. Treatment is supportive, with advice to avoid both alcohol (particularly if hepatitis is present) and vigorous exercise, the latter because of the risk of splenic rupture. Steroids are reserved for serious complications and, if required, a short reducing course is usually adequate (McGowan, Jr. et al., 1992). Aciclovir inhibits viral replication but is ineffective in altering the clinical course of IM (Tynell et al., 1996). This is because the clinical symptoms are not due to viraemia, but to the marked immune response to EBV, which is well established by the time of clinical presentation.

Relapses can occur in the first 6-12 months following infection, and IM may be linked, in the short term, with the development of a prolonged fatigue syndrome and depression (White et al., 1998). The risk of these complications seems to be higher in

those with pre-morbid psychological and social problems and lower physical fitness (White et al., 2001). However, there is no evidence that the chronic fatigue syndrome is caused by abnormal immunological response to EBV (Swanink et al., 1995).

Little data are available on the severity or length of fatigue in the immediate period following acute IM, although this may be one of the most disabling symptoms. Assessment of fatigue in an objective manner is difficult; particularly as the majority of fatigue scales described in the literature are pertinent to either an older age group or a specific condition such as post chemotherapy or pregnancy (Monga et al., 1999).

2.6.7 Conditions associated with life threatening primary EBV Infection

A number of conditions have been linked to severe or fatal EBV infection, and X linked proliferative disease (XLP) is the most well known. XLP, and the other conditions are discussed in detail following the section on immune response to primary EBV infection (See section 2.8 page 50).

2.6.8 Immune response to primary EBV infection

The key feature of the immune response to IM is the marked expansion of atypical lymphocytes, and much progress has been made in characterising the scale, phenotype and epitope specificity of these cells. Moreover, this work in IM has provided a useful model for studying the immune response to other persistent viral infections in humans. Much of this progress has been made by studying, ex vivo peripheral blood mononuclear cells (PBMC) from young adults with IM, and accepting this is a good model of primary infection. However it should be recognised that there are some fundamental limitations to this work. Studies on silent seroconvertors, either as adults or young children are very limited, but silent seroconversion is the most common mode of EBV acquisition. The other caveat is that very limited data are available on immune responses in the tissues and lymph nodes where much of the pathology of IM occurs.

2.6.8.1 A CD8+ lymphocytosis dominates the immune response to IM

The majority of PBMC found in the peripheral blood in IM are activated lymphocytes and typically the majority (over 70%), are CD8+ T cells, which express

the activation markers HLA-DR+ CD45RO+CD45RA (Tomkinson et al., 1987; Callan et al., 1996; Hoshino et al., 1999). This dramatic CD8+ T cell response is thought to both control the infection by lysis of proliferating EBV infected B cells, and cause the symptoms of IM by excess cytokine release.

The dramatic scale of this CD8+ lymphocytosis has promoted debate as to whether these cells are superantigen driven, or are a large clonal response to a specific viral antigen (Sutkowski et al., 1996). Recent work is widely accepted as identifying the majority of these cells as antigen specific, both by analysis of T cell receptor (TCR) diversity and use of tetramer technology (Callan et al., 1996; Callan et al., 1998).

Firstly IM patients were shown to have expansions of CD8 cells that used a variety of T cell receptor (TCR) variable region beta chains (Vβ) (Callan et al., 1996), whereas the response to a superantigen would predict selective use of Vβ chains (Sutkowski et al., 1996). Moreover, TCR Vβ chains selected by the same antigenic peptide in the groove of the MHC molecule would tend to show sequence conservation in the Vβ chains. Analysis of the sequence of the expanded TCR Vβ chains in individuals with acute IM showed such sequence conservation. Again, this confirmed the oligoclonal nature of the expansions, consistent with an antigen specific response. Subsequently, the advent of tetramer technology allowed the detection of CTL specific to antigenic epitopes of the investigator's choice. Studies in acute IM showed dramatic expansions of antigen specific CTL; in one case 44% of the total CD8+ cells within the peripheral blood were specific for a single lytic epitope of EBV (Callan et al., 1998).

Further work studying the antigen specificity of CTL in acute IM, has shown that the immunodominant epitopes used are predominantly from immediate early (BZLF1 and BRLF1), with some from early proteins (BMLF1, BMRF1, and BALF2) of the lytic cycle (Silins et al., 1996; Elliott et al., 1997; Steven et al., 1997; Hislop et al., 2002). CTL responses to the late proteins which encode the structural antigens gp350 and gp85 are much weaker (Khanna et al., 1999b). In addition, these studies showed that the frequency of antigen specific cells fell following recovery from IM; however these cells continued to be detectable up to three years after infection (Silins et al., 1997). The frequency of CD8+ T cells reactive to the latent antigens appears to be

much lower. Callan et al found 1-2 percent of the total CD8+ T cell population was directed against the EBNAs (Callan et al., 1998), whereas in a further study Hislop et al, found responses against LMP-2 were very rare (Hislop et al., 2002).

The role of CD4 + T cells in the control of primary infection is less well understood. The most detailed investigation of CD4+ T cells responses in IM identified cells specific to both lytic and latent antigens. Like CD8+ T cell responses, the most frequently detected CD4+ cell responses were also to the lytic antigens, (in this case BZLF1), compared to the latent antigens studied (EBNA -1 and EBNA- 3A) (Precopio et al., 2003). One other study identified CD3+CD4+CD8+ lymphocytes which were reactive against the lytic cycle antigen BHRF-1 (White et al., 1996). Data are limited on the response of other lymphocyte subsets during primary infection, although expansions of both NK (Tomkinson et al., 1987), and γδ cells have also been identified (Hassan et al., 1991; De Paoli et al., 1990).

2.6.8.2 The cytokine response is critical to the pathogenesis of IM

Cytokines are thought to be critical to the pathogenesis of IM, and the release of excessive T helper 1 type cytokines is thought to cause the typical clinical features. The best evidence to support this derives from studies of cytokine gene expression in tonsil from IM patients: expression of mRNA for lymphotoxin, tumour necrosis factor α (TNF α), and Interleukin (IL)– 6 have all been detected (Foss et al., 1994). In addition, several investigators have found elevated levels of IL-2, IL-6, TNF α and IFN γ in the peripheral blood at diagnosis of IM (Biglino et al., 1996; Andersson et al., 1984), and the same cytokines have been identified after in vitro infection of PBMC with EBV (Andersson et al., 1984).

It remains difficult to ascertain how this cytokine network evolves in vivo, however it is probably reasonable to postulate that the earliest events are the release of IL-6 and TNF α either from monocytes or infected B cells. IL-6 can act on both B and T cells; it induces the differentiation of B cells, and both proliferation by T cells and release of IL-2 by T cells (Hirano et al., 1990). In addition, TNF α is an important activator of NK cells (Cooper et al., 2001a). In terms of symptom causation, both NK cells and activated T cells are an important source of INF γ, a key candidate,

alongside IL-6 and TNF α for causing the systemic features of IM such as fever (Dinarello, 1999).

2.6.8.3 The humoral immune response to EBV

During primary infection antibodies are generated to both lytic and latent viral antigens. Neutralising antibody to gp 350, the main viral envelope glycoprotein, and also gp85 another glycoprotein on the viral envelope prevent binding to the CR2 receptor on B cells in vitro (Tanner et al., 1987). It is not clear whether these antibodies have a role in limiting primary infection, but are thought to prevent re-infection. Currently EBV vaccines based on the generation of neutralising antibody are being developed (Jackman et al., 1999). For further details of vaccines see 2.9 page 52.

2.6.8.4 Symptomatic versus asymptomatic primary EBV infection

Despite our increased understanding of immune response to EBV, it is not clear why the manifestations of primary infection are different between age groups and individuals, and how the scale of the immune response relates to clinical features. It is likely that milder forms of the syndrome occur without detection, and a spectrum of clinical features exists ranging from mild sore throat to full blown IM. Some insight has been gained into the mechanisms of silent infection, as 3 cases of asymptomatic seroconversion have been fortuitously identified during a phase 1 vaccine study in Australia (Silins et al., 2001). In contrast to those with IM, the silent seroconvertors did not develop the lymphocytosis or increased CD8+ cells count typical of IM. The use of T cell repertoire was also analysed and there was no change in usage between primary and persistent infection in the symptomatic group, whereas the IM cases showed marked expansions in the spectrum of T cell receptors used. The authors suggest that that this identified expansion of T cells in acute IM supports the hypothesis that the pathology of IM is modulated by the T cell response. In addition although the numbers studied were small, EBV load was compared between the 2 groups, and 2 out of 3 of the asymptomatic cases showed levels equal or higher to the 3 symptomatic cases.

Additionally it has been suggested that infants are more likely to silently seroconvert as they receive a lower dose of infecting virus than young adults (Epsten MA Crawford DH, 1996). This is because transmission in infants is likely to be from close family contact, such as sharing toys or cups, compared to sexual contact by young adults. The other important factor is potential differences in the immune response to infection between children and young adults. This is likely to be relevant as children manifest different clinical responses to a number of other infectious agents such as VZV and parvovirus.

These hypotheses are difficult to investigate in vivo in humans, and an animal model would be useful in investigating the pathogenesis of IM. One such model is the murine gamma herpes virus, MHV-68 (gamma HV-68) infection of mice in which the virus establishes latency in B cells (Nash et al., 2001). However, a primate may provide a better model for vaccine studies and rhesus monkeys are infected by EBV-like lymphocryptovirus. This results in an IM like syndrome following primary infection, and importantly the virus is secreted in the oropharynx (Wang, 2001).

2.6.9 The immune response to persistent infection

After acute infection a state of viral persistence is established with long term cellular immunity provided by EBV-specific CD8 + cytotoxic T lymphocytes. These cells can be detected in the circulation in all healthy seropositive individuals, and normally have a resting phenotype (HLA DR-CD38-CD62+) (Tan et al., 1999).

As suggested by the studies during recovery from IM, the CTL detected in latency are directed against both lytic (BZLF1 and BMLF1) (Steven et al., 1997), (BRLF1) (Pepperl et al., 1998) and latent viral epitopes (EBNA3A, EBNA3B, and EBNA3C) (Murray et al., 1992) and reviewed in detail by (Khanna and Burrows, 2000).

CD4+ T helper cells have a central role in the maintenance of antigen specific immunity and in mouse models are important in resistance to both virus infection and cancer (Bickham et al., 2001). Like studies during acute infection, studies on persistent infection in healthy virus carriers have focused on identification of CD8+ T cell responses, rather than CD4+ T cells. This may be due, in part, to the difficulties of identifying CD4+ T cells specific for EBV antigens; tetramer

technology is not yet well established for the identification of CD4+ T cell responses (Nepom et al., 2002).

However several groups have identified EBNA-1 responses in the majority of donors, and additionally one group found a minority of donors, around 10%, to have responses to LMP-1 and LMP-2 (Munz et al., 2000; Bickham et al., 2001) .

Of particular interest is the identification of EBNA-1 specific cells, as EBNA-1 (Gly-Ala) repeat interferes with processing and presentation by CD8+ T cells (Levitskaya et al., 1995). Although EBNA 1 specific CD8+ T cells can be recognised in vivo, they require exogenous antigen to allow for presentation in the context of HLA1 (Blake et al., 1997).

2.7 X-linked lymphoproliferative disease (XLP)

2.7.1 Duncan's syndrome and the XLP registry

In 1998, two groups, taking quite different approaches, identified the genetic defect underlying the rare X linked lymphoproliferative disease XLP (Coffey et al., 1998; Sayos et al., 1998). The syndrome was first described by Purtilo and colleagues in 1975, although both Bar and Purtilo had previously identified similar cases (Bar et al., 1974; Purtilo et al., 1974; Purtilo et al., 1975; Bar et al., 1974). Purtilo recognised that a novel X linked genetic defect was likely to be the cause of the severe and fatal lymphoproliferation, which had been documented in 6 out of 18 male members of the Duncan family. In at least 3 of the 6 boys, IM had occurred during or prior to death, and 2 of the 6 boys also developed lymphoma. Purtilo called the syndrome Duncan's Disease, and correctly suggested EBV or other viral agents were the trigger for the syndrome.

In 1976, with the identification of further cases the syndrome was named X-linked lymphoproliferative syndrome (XLPS) (Schuster et al., 1993) and in 1978 a registry was established (Hamilton et al., 1980). The name was changed to X-linked lymphoproliferative disease (XLP) in 1990 when a single XLP gene locus on Xq25 was identified (Wyandt et al., 1989b). In 2002 data had been recorded from 309 males from 89 families, and XLP was reported to affect around one in a million

males (Sumegi et al., 2000). The collection of data by the registry has allowed important analysis of the spectrum of clinical features that occur in XLP, and diagnostic criteria to be established. The registry now maintains a website at www.hta.fi/imt/bioinfo/idr/Diag_XLP.html.

2.7.2 Clinical Manifestations of XLP

The mean age of onset of XLP is 2.5 years, however cases have been observed as young as 5 months and up to 40 years. Three major clinical phenotypes are recognised (Seemayer et al., 1995) (Table 2-7 page 40), however individuals may suffer from a combination of these phenotypes.

Table 2-7 Common clinical phenotypes of XLP (Seemayer et al., 1995).

Phenotype	Frequency	Mortality of each phenotype
Fulminant IM	58%	96%
Lymphoma (mostly extranodal B cell origin)	30%	69%
Hypogammaglobulinemia	31%	45%

The most common phenotype is fulminant IM, which occurs in around two thirds of cases, and has the worst prognosis with a mortality rate of over 90%. In response to primary EBV infections the affected boys rapidly develop large numbers of EBV infected B cells which in turn stimulate a dramatic proliferation of cytotoxic T cells. At post mortem large numbers of EBNA positive cells have been found in the blood and infiltrating the tissues (Crawford et al., 1979). Unlike the normal response to EBV, the T cells are apparently unable to limit the proliferation of B cells, and this dysregulated cytotoxic T cell response and subsequent cytokine release leads to extensive organ damage (Purtilo et al., 1975; Seemayer et al., 1995). Bone marrow failure remains the most common cause of death in XLP (Schuster and Kreth, 1999).

The second most common phenotype is hypogammaglobulinemia, which is found in a third of cases. Again the aetiology is likely to be immune mediated with subsequent necrosis of the lymphoreticular system. Affected individuals have low IgG and IgA, and low to normal IgM (Grierson et al., 1991; Seemayer et al., 1995). T and NK cell

numbers may also be low, and have defective function (Sullivan et al., 1980; Harada et al., 1982; Argov et al., 1986; Masucci et al., 1981). Boys suffering from hypogammaglobulinemia may be treated with regular immunoglobulin infusions and in isolation this phenotype carries the most favourable prognosis. Since identification of the gene, a number of men with common variable immunodeficiency have been found to, in fact, have mutations in the XLP gene (Gilmour et al., 2000; Soresina et al., 2002).

The other major phenotype is lymphoma and these are normally of B cell origin (Grierson and Purtilo, 1987). The most common site is the terminal ileum, but also hepatic, renal and central nervous system lymphomas have been described. A small number of cases of Hodgkin's disease and T cell lymphomas have also been reported (Harrington et al., 1987). Of note, lymphomas of different subtypes have occurred within the same family (Hamilton et al., 1980). The tumours may respond to standard paediatric chemotherapy protocols, but the mortality remains high at 69% (Gaspar et al., 2002).

It is not clear what proportion of lymphomas in XLP contain EBV, although there is evidence that lymphoma may develop without serological evidence of prior EBV infection (Brandau et al., 1999).

Other manifestations of XLP include systemic vasculitis (Dutz et al., 2001), aplastic anaemia (Poy et al., 1999; Seemayer et al., 1995), and pulmonary lymphomatoid granulomatosis (Gaspar et al., 2002).

2.7.3 Immunodeficiency prior to EBV exposure

Affected boys are typically reported as being well until encountering EBV, but there is some debate as to whether immunological abnormalities occur prior to EBV infection. This is a difficult area to study, firstly asymptomatic cases can only be identified through screening family members of affected individuals, and secondly there is growing evidence that XLP is not only precipitated by EBV infection. However, immunological studies on XLP cases in the 1980's suggested that boys with XLP frequently do not develop full antibody memory, characterised by an inability to switch from IgM to IgG isotype (Ochs et al., 1983). This is especially

interesting, as recent work on a mouse model of XLP has confirmed this finding (Crotty et al., 2003), and this is reviewed in the discussion (See section 9.4 page 172).

In 1995 Seemayer reported on 32 cases from the registry who had been identified as carrying the mutated gene by restriction fragment length polymorphism but whom had no serological evidence of EBV infection. Of the 32 cases identified, 17 in fact fulfilled diagnostic criteria for XLP as they had dysgammaglobulinemia, with raised IgA and IgM and/or defects in IgG, IgG1 or IgG3 production (Seemayer et al., 1995).

2.7.4 Treatment of XLP

The treatment of XLP remains difficult, and the only curative option is allogeneic haemopoietic stem cell transplantation (Vowels et al., 1994; Pracher et al., 1994; Gross et al., 1996). The most common presentation, fulminante IM remains the most difficult to treat; use of the anti-histiocyte agent, etoposide may induce remission prior to bone marrow transplant. The rarity of the condition and diverse clinical spectrum of presentations makes any comparative analysis of treatment options difficult, and survival rates remain extremely variable (Gaspar et al., 2002) . Genetic screening of male family members is now possible, and regular immunoglobulin infusions may protect those identified as at risk of developing clinical XLP.

2.7.5 Identification of the XLP gene

The first breakthrough in the search for the gene was in 1989, when the locus for XLP was linked to the long arm of the human X chromosome in Xq24-q27 (Skare et al., 1989a; Skare et al., 1989b). Further deletions at this site in other XLP cases provided confirmatory evidence that this was the XLP locus, and narrowed it down to Xq25 (Wyandt et al., 1989a). Coffey et al working with an international consortium, identified the gene using a positional cloning strategy with XLP patients with known deletions as reference points (Coffey et al., 1998). The critical region was localised to an approximately 3Mb region, and a yeast artificial chromosome contig was established to provide a physical map of the region. Four candidate genes

were identified using exon trapping and sequencing techniques. Only two of these genes were candidates, as the other two were found to lie partly outside the critical region. Further refining of the critical region led to the identification of the likely candidate as a gene which encoded a SH2 domain. This gene, subsequently named SH2D1A, was mutated in 9 out of 16 unrelated XLP cases, but not in healthy controls.

The SH2D1A gene contains 4 exons. It encodes a protein of 128 amino acids, which contains a 5-amino acid N terminal sequence, an SH2 domain, and a 25-amino acid C terminal tail (Coffey et al., 1998; Poy et al., 1999).

Sayos et al cloned the XLP gene serendipitously, when they identified a signalling protein for the cell surface lymphocyte activation marker termed SLAM (Sayos et al., 1998). This novel SH2 domain containing protein was called SLAM associated protein (SAP). SLAM/CD150 is a T/B/dendritic cell cell surface glycoprotein, and is a costimulatory molecule involved in cellular activation. The mouse homologue of SAP was found on the mouse X chromosome, at a point corresponding to human Xq25. Mutations in SAP were subsequently detected in 3 XLP patients, but not in over a hundred healthy controls. Moreover, the mutant SAP proteins where unable to bind to SLAM. Identification of these mutations, in a novel T cell activation pathway provided the first insight into the molecular pathophysiology of XLP.

Morra and colleagues have suggested the XLP gene should be called SAP/ SH2D1A and the gene product SAP (Morra et al., 2001).

2.7.6 The relationship between mutations identified in XLP and clinical phenotype

Since the identification of the gene a number of groups have looked for mutations in the gene in clinically diagnosed cases of XLP. Sumegi and colleagues have screened the largest number of affected families, 35 from the XLP registry, for mutations in the SAP gene, and looked for any relationship between genotype and clinical phenotype (Sumegi et al., 2000). They identified 28 different mutations and classified them as large genomic deletions of the entire gene or part of the coding region, splice-site mutations and nucleotide substitutions, both nonsense and mis-

sense. The most common mutations were large genomic deletions. No relationship was found between genotype and phenotype, and in particular those with large genomic deletions did not appear to have worse disease than those with single nucleotide substitutions. It was also noted that among families with the same mutation very variable clinical presentations may occur, providing further evidence that additional environmental or virological factors must contribute to the clinical outcome.

At least another nine series of mutation analysis of XLP patients have been published (Coffey et al., 1998; Nichols et al., 1998; Yin et al., 1999; Strahm et al., 2000; Brandau et al., 1999; Nistala et al., 2001; Arico et al., 2001; Gilmour et al., 2000; Sumazaki et al., 2001). All groups used PCR based sequencing techniques. Mutations have most frequently been identified in exon 2, particularly nucleotide substitutions or deletions. This is functionally significant as it is exon 2, which encodes the central portion of the SH2 domain, which is critical to the function of SAP. One hot spot has been identified, at nucleotide position 462 resulting in an Arg55stop transition. 3 different groups have described the same mutation, however it is possible that more than one investigator has inadvertently analysed DNA from the same affected individual. Otherwise mutations are sporadic throughout the four exons. In most series around 50-60 % of those with a clinical diagnosis of XLP have been found to have mutations in SAP/SH2D1A. Predictably, more mutations have been identified in cases with a strong family history compared to sporadic cases of fulminant IM.

Several investigators have looked for mutations in SAP in other conditions in which severe EBV infection occurs. Two groups have looked for mutations in a total of 26 cases of chronic active EBV (CAEBV) (Sumazaki et al., 2001; Sumegi et al., 2000) and no mutations in SAP/SH2D1A were found. In a study of 25 cases of hemophagocytic lymphohistiocytoses (HLH), 2 out of 25 cases were found to have mutations in SAP (Arico et al., 2001).

2.7.7 Cellular and tissue distribution of SH2D1A/SAP

SAP is predominantly expressed in T lymphocytes (Sayos et al., 1998), and NK cells (Tangye et al., 1999). The T cell subsets include both CD4+ and CD8+ cells, and the

CD8+ subsets were also noted to express CD45RO+ and CD45RA+. Although SAP is detected in resting lymphocytes, expression is increased on activation (Nagy et al., 2002). Nagy et al cultured PBMC either with EBV or PHA and found increased expression of SAP in the cultured cells compared to ex vivo PBMC. In addition tumour cell lines derived from both T lymphocytes and NK cells were found to express SAP, like their cellular counterparts (Kis et al., 2003).

Coffey et al found expression of SAP mRNA in B cell subsets (Coffey et al., 1998), however other investigators have not confirmed this finding, and the consensus opinion is that B cells do not express SAP protein (Sayos et al., 1998; Nichols et al., 1998; Gilmour et al., 2000). It is possible that the B cell subset tested by Coffey et al was not an entirely pure population of cells, as no comment is made on the cell separation method used. B cell derived LCL's have also been found to be consistently negative, though Nagy et al have found expression of the protein in a number of EBV positive BL derived cell lines, though not EBV negative BL lines (Nagy et al., 2000). The reasons for the expression in BL derived lines is unclear. A limited survey has been carried out of SAP/SH2D1A expression in lymphoma tissue; Hodgkin's and non-Hodgkin's lymphoma of both T and B cell origin expressed the gene (Nichols et al., 1998).

Both Coffey and Sayos who identified the SAP/SH2D1A gene surveyed human tissue for expression of SAP, using Northern blotting (Coffey et al., 1998; Sayos et al., 1998). Both groups found the highest expression of SAP in the thymus, but the gene was also expressed in the spleen, the lungs and liver. SAP expression was not identified in prostate, testis, ovary, intestine, colon or brain. This pattern of gene expression in the tissues is consistent with a T cell protein.

2.7.8 SAP and regulation of cell surface lymphocyte activation molecules SLAM and CD244

SAP is a small protein in which the first three exons encode the SH2 domain and exon 4 encodes the tail sequence. Immediately upstream of exon 1 are putative binding sites for transcription factors involved in T cell development and function (Sayos et al., 1998; Sayos et al., 2000). The crystal structure of SAP has been published (Poy et al., 1999).

SAP was first identified as a cytoplasmic signal transduction protein for cell surface SLAM (Sayos et al., 1998), and its function is now thought to be as a cytoplasmic regulator of cellular activation by at least a further 4 cell surface receptors (Tangye et al., 1999). The structure of the protein, which lacks signalling domains, predicts that it functions as a blocking molecule (Poy et al., 1999). By binding to phosphorylated SLAM, SAP prevents signalling by other SH2 domain containing proteins (such as SHP-2), which do have down stream signalling capacity (Figure 2-1 page 46). As well as SLAM, SAP binds 2B4/CD244 (Tangye et al., 1999), CD84 and Ly-9 (Sayos et al., 2001), and NTB-A (Bottino et al., 2001). All are members of the immunoglobulin gene super family and belong to the CD2 family of cell surface receptors.

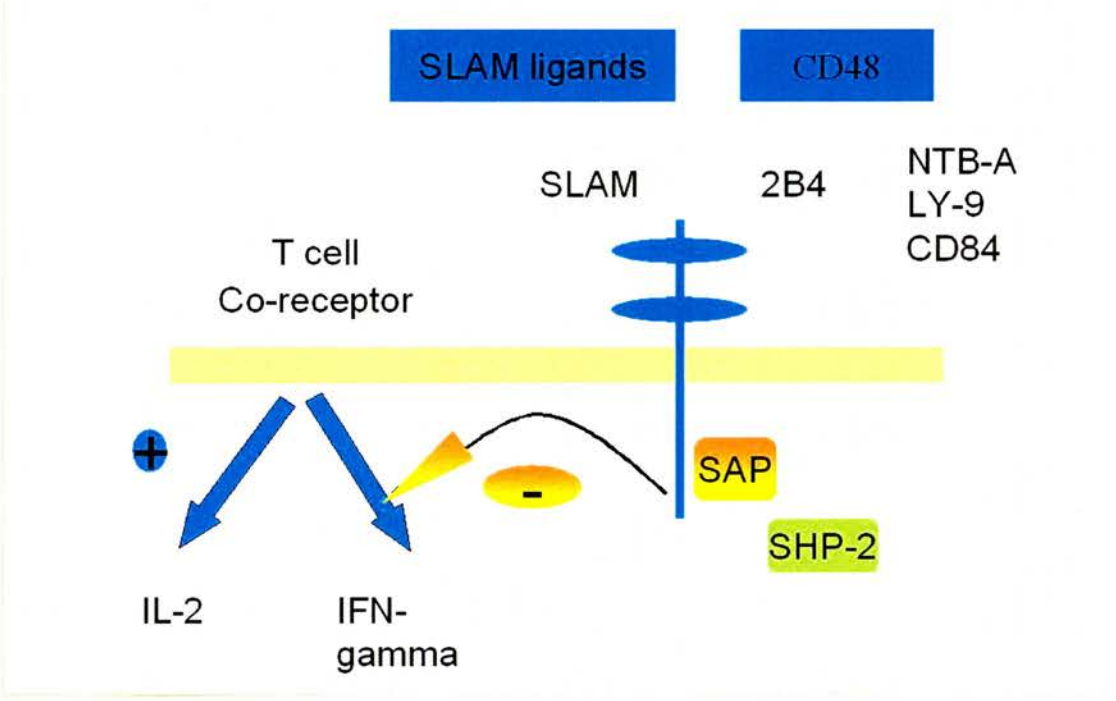


Figure 2-1 SAP transduction pathway

2.7.8.1 SLAM/CD150

SLAM is primarily expressed on lymphocytes and expression has been documented on resting T cells, B cells and dendritic cells; expression is rapidly upregulated on both CD4 + and CD8+ subsets following activation in vitro (Aversa et al., 1997). Engagement of SLAM by specific antibody leads to T cell proliferation, and preferential production of TH1 type cytokines including IFN γ (Garcia et al., 2001).

SLAM is also expressed on B lymphocytes and in vitro studies have suggested that signalling through SLAM leads to proliferation of B cells and immunoglobulin synthesis, of IgM, IgG and IgA subclass (Punnonen et al., 1997). In addition to the membrane bound form of SLAM, activated lymphocytes express mRNA for a secreted form (Punnonen et al., 1997). Studies in human disease have documented increased expression of SLAM in lymphocytes from synovial fluid of patients with both rheumatoid arthritis and reactive arthritis compared to lymphocytes from the peripheral blood (Isomaki et al., 1997). SLAM can function as a self ligand, however other ligands have not yet been identified.

Also of interest, SLAM has been identified as a receptor for wild type measles virus (Tatsuo et al., 2000), and this finding may eventually provide some insight into the pathogenesis of measles associated immunosuppression.

2.7.8.2 2B4/CD244

Like SLAM human CD244 is a cell surface lymphocyte receptor which is phosphorylated following activation and recruits SAP (Tangye et al., 1999). Human CD244 is expressed on all NK cells and on around 50% of CD8+ lymphocytes. It is not expressed on resting B cells or CD4+ lymphocytes (Nakajima et al., 1999; Kubin et al., 1999). Cross linking of CD244 on NK cells results in enhanced cytotoxicity and cytokine production including IFN γ (Nakajima et al., 1999).

There has been particular interest in the role of CD244 in the pathogenesis of XLP as CD244 is the counter receptor for the B cell activation molecule CD48 (Latchman et al., 1998). CD48 is expressed at ten times higher levels on EBV infected B cells than EBV negative cells (Thorley-Lawson et al., 1982). Therefore this interaction between CD48 and CD244 may provide a important mechanism by which both CD8+ T cells and NK cells recognise EBV infected B cells in vivo.

Four groups have shown that NK cells from XLP patients maintain normal expression of CD244, but fail to initiate cytotoxicity via this receptor (Parolini et al., 2000; Benoit et al., 2000; Tangye et al., 1999; Nakajima et al., 2000). NK cell activity via other receptors such as CD16, which does not associate with SAP, was found to be normal. Of particular interest, Parolini and colleagues showed that NK

cells from XLP patients were unable to kill either autologous or allogeneic LCL. However, killing was restored by simultaneous disruption of 2B4-CD48 and NK receptor-MHC 1 interactions. Parolini, unlike the other investigators, did not only find that NK cells from XLP patients failed to kill via CD244, but that engagement of CD244 actually inhibited killing by NK cells. This failure of CD244 mediated killing in XLP may explain the defects in NK cell killing previously identified in XLP patients (Argov et al., 1986; Harada et al., 1982; Masucci et al., 1981; Sullivan et al., 1980; Sullivan et al., 1980). A further receptor, NTB-A, also binds to SAP and has a very similar function to CD244. NTB-A is expressed on NK and T cells, but unlike CD244 is also expressed on B lymphocytes (Bottino et al., 2001).

2.7.8.3 Ly-9 and CD84

Ly-9 (CD299) and CD84, two further cell surface receptors, have also been found to bind SAP via their cytoplasmic tails (Sayos et al., 2001). Like the other receptors in the family, Ly-9 and CD84 are expressed on both T and B lymphocytes.

2.7.8.4 SAP transduction pathways

Identification of the XLP gene product SAP has generated a great deal of interest in the role of SAP in controlling T and NK cell activation pathways. Much of this work has focused on a role for SAP as a natural inhibitor of T cell activation, initiated by ligation of the SLAM family of TCR co-receptors (Sayos et al., 2000; Morra et al., 2001). On the other hand, work by Latour and colleagues, suggests SAP is not just an inhibitory molecule but may facilitate recruitment of FynT tyrosine kinase to the phosphorylated cytoplasmic domain of SLAM (Nichols et al., 2001; Latour et al., 2001). The recruitment of FynT leads to a signaling cascade which subsequently inhibits secretion of IFN γ by activated T cells. The cytokine response in activated T cells, which lacked either SAP or SLAM was particularly interesting. In cells that expressed both SLAM and SAP, TCR ligation inhibited INF γ secretion, but did not affect IL-2 production. This finding highlights the importance of SAP expression to the control of INF γ, a cytokine that is thought to be critical to the pathogenesis of both IM and XLP. However, the results of this in vitro work should be interpreted in

the context of our knowledge that normal individuals without defects in SAP or SLAM can express INF γ from activated T cells.

In contrast, ligation of CD244 and CD84, both expressed on NK cells, and also acting via SAP, results in secretion of INF γ. This activation cascade is thought to involve signalling via phospholipase Cγ and linker for activation of T cells. In summary, SAP controls several distinct signalling pathways in T and NK cells, all of which may modulate cytokine production (Engel et al., 2003).

Mouse models of XLP, in which mice with defects in SAP expression have been generated, have corroborated the immunological findings which have been documented humans with XLP (Czar et al., 2001). In response to lymphocytic choriomeningitis virus (LCMV) increased numbers of CD4+ and CD8+ lymphocytes, which produced INF γ, were observed. Interestingly, even prior to virus infection, a skewing of the lymphocyte cytokine profile towards a TH1 phenotype, and defective IgE antibody production was observed (Czar et al., 2001).

2.7.9 Improved screening for XLP and Diagnostic Criteria (Defined by the XLP Registry)

The identification of the XLP gene has allowed a definitive diagnosis to be made in putative XLP cases. New criteria for the diagnosis of XLP have been proposed by the European Society for Immunodeficiencies (ESID) and Pan American Group for immunodeficiencies (PAGID) and are documented below (Conley et al., 1999).

2.7.9.1 Definitive XLP

Male patient with lymphoma/Hodgkin's disease, fatal EBV infection, immunodeficiency, aplastic anemia or lymphohistiocytic disorder who has a mutation in SH2D1A/SAP/DSHIP.

2.7.9.2 Probable XLP

Male patients experiencing death, lymphoma/Hodgkin's disease, immunodeficiency, aplastic anaemia or lymphohistiocytic disorder following acute EBV infection and

maternal cousins, uncles or nephews with a history of similar diagnosis following acute EBV infection.

2.7.9.3 Possible XLP

Male patients experiencing death, lymphoma/Hodgkin's disease, immunodeficiency, aplastic anaemia or lymphohistiocytic disorder following acute EBV infection. This is sometimes described as sporadic XLP.

2.8 Other conditions associated with severe primary EBV infection

The identification of the XLP gene, as well as improved techniques in immunophenotyping and molecular genetics, has increased our understanding of the spectrum of conditions in which primary EBV infection is life threatening. A number of syndromes are recognised which occur in previously immunocompetent individuals (Table 2-8 page 50). These include sporadic fulminant IM, which may be linked with EBV associated haemophagocytic syndrome, fulminant EBV+ T–cell lymphoproliferative disorder, and chronic active EBV (CAEBV). Both EBV associated haemophagocytic syndrome (Imashuku, 2002), and fulminant EBV+ T – cell lymphoproliferative disorder (Quintanilla-Martinez et al., 2000) are pathological classifications which present as fulminant IM, whereas CAEBV is recognised as a distinct clinical entity (Kimura et al., 2001).

Table 2-8 Conditions associated with life threatening EBV infection

Disease or syndrome	Clinical Presentation	Key diagnostic feature
XLP	Life threatening IM, hypogammaglobulinaemia, atypical lymphoma	Mutations in SAP/SH2D1A
EBV associated haemophagocytic syndrome	Life threatening IM	Haemphagocytosis in bone marrow
Sporadic fulminant IM	Life threatening IM	No mutations in SAP/SH2D1A
Fulminant EBV+ T –cell lymphoproliferative disorder	Life threatening IM	Clonal expansions of EBV infected T cells.
CAEBV	Recurrent severe IM	Minimum of 6 month course Very high viral load T or NK cells infected with EBV

Fulminant IM occurs sporadically, in both males and females, as well as in those with defects in SAP/SH2D1A underlying XLP.

2.8.1 EBV associated haemophagocytic syndrome

The diagnosis of this syndrome is made histologically and is characterised by the presence of phagocytosis of blood cells in the bone marrow. EBV infection is thought to be one of the most common virological causes of haemophagocytosis, particularly in childhood (Ohshima et al., 1999). However a number of other viruses have been implicated including hepatitis A (Ishii et al., 2003), as well as non viral causes such as malignancy.

Clinically haemophagocytosis presents in a similar manner to fulminant IM and haemophagocytosis has been described in XLP (Arico et al., 2001).

2.8.2 Fulminant EBV+ T–cell lymphoproliferative disorder

Fulminant EBV+ T–cell lymphoproliferative disorder is a very rare disorder, which may present following primary infection, but also occurs in association with CAEBV (Quintanilla-Martinez et al., 2000). The key pathological feature is clonal expansion of EBER+CD3+T cells, which typically infiltrate the liver and spleen. A recent case series and review has helped define the syndrome and suggests the prognosis is extremely poor; of a total of 16 cases described in the literature 15 were dead within a year. The sudden clinical onset with rapid deterioration, and presence of large numbers of EBV positive T cells, is suggestive of acute infection, however surprisingly EBV serology may not be typical of primary infection.

2.8.3 Chronic Active EBV (CAEBV)

CAEBV is characterised by chronic or recurrent IM-like symptoms which are life threatening. Typically, the clinical course is less acute than fulminant IM, but the longterm prognosis is poor. The disease is most common in Japan, and a recent national survey carried out by Kimura and colleagues has helped to clarify the key features (Kimura et al., 2001; Kimura et al., 2003). The diagnostic criteria for CAEBV used in the survey, included a greater than 3 month duration of EBV related illness, a very high number of EBV genome copies in the peripheral blood or a

grossly abnormal antibody profile. In total, they identified 82 patients who fulfilled these criteria and had been diagnosed since 1990.

Like XLP, CAEBV predominately occurs in children, and Kimura found the mean age of onset was 11 years with a range from 9 months to 53 years. The mortality was high, at over 40%, with a mean survival of only 4.3 years from onset. The causes of death included hepatic failure, complications of transplant, lymphoma and hemaphagocytic syndrome. Kimura also made an important observation that unlike healthy virus carriers, T and NK peripheral blood lymphocytes are infected with EBV, and the type of cell infected could be linked to prognosis. Those with T cell CAEBV had a shorter survival time than those with NK cell disease. Another prognostic feature was the EBV DNA viral load in PBMC, with higher viral loads found in those with severe disease. This data may be useful in guiding treatment options. Bone marrow transplant may be successful (Okamura et al., 2000; Kawa et al., 2002), but should be reserved for those with a poor prognosis as the risk of transplant is substantial. Another new treatment option is infusion of EBV specific CTL (Savoldo et al., 2002).

2.9 Vaccine Development

Both primary and persistent EBV infections are associated with significant pathology and this has led to interest in the development of an EBV vaccine. An ideal vaccine would prevent the complication of late primary infection, IM, and prevent viral persistence, thereby potentially eradicating EBV driven cancers. At present there are two main approaches to vaccine development (reviewed by (Khanna et al., 1999a).

First, induction of neutralizing antibody to gp 350 should prevent virus binding to CD21, the key B cell receptor, and thus prevent infection. Gp 350 vaccines are under trial, but it is not clear yet whether induction of an antibody response, possibly with induction of cytotoxic T cells to the same viral epitope, will provide sterile immunity (Khanna et al., 1999b). The first trial to be reported was a small Chinese trial of 19 children, 9 who whom received a live recombinant vaccinia virus expressing Gp350 (Gu et al., 1995). Of these 9 children, 6 remained anti-VCA negative sixteen months after vaccination, compared to the 10 controls who all

seroconverted during this period. Although these results are promising, use of live vaccinia virus vaccines is generally considered unacceptable by the licensing authorities.

The second approach to vaccination is the induction of cytotoxic T cells specific to EBV by vaccination with synthetic peptides (Moss et al., 1998). As cytotoxic T cells are critical to controlling primary infection, this mechanism may limit early viral replication. This in turn may significantly attenuate the immune response to primary infection, and the associated clinical features of IM, but probably would not prevent establishment of viral persistence. The major difficulty with this type of vaccine is the need for multiple CTL epitopes to be combined within the vaccine. This is because each MHC allele presents a different viral epitope, and so a cocktail of epitopes must be included to cover the diversity of MHC alleles within a population. However we now know that certain groups of MHC class 1 molecules bind very similar peptides (Thomson et al., 1995), thus exploitation of these MHC 'supertypes' may allow a significant proportion of the population to be covered by a relatively small number of peptides.

CTL based vaccines are also being developed as therapeutic vaccines against EBV linked tumours (Duraiswamy et al., 2003). Recent work has focused on the development of an LMP1 specific vaccine, as LMP-1 and LMP-2 are the key latent antigens expressed in EBV- associated nasopharngeal carcinoma and Hodgkin's disease (Lee et al., 1997; Duraiswamy et al., 2003; Meij et al., 2002).

Even if an ideal vaccine is developed there will be considerable difficulties in deciding when to use it and in whom to use it. As previously discussed the data on the epidemiology of IM is several decades old, and up to date information is required in order to identify the target population, and the appropriate age to offer vaccination. Perhaps with the recent advent of vaccines to human papilloma virus and herpes simplex virus (Koutsky et al., 2002), vaccination against sexually transmitted disease prior to puberty will become more acceptable. Other small target populations are likely to be seronegative transplant recipients and possibly boys with mutations in SAP/SH2D1A prior to infection with EBV.

2.10 Human NK cells

CTL are recognised as the major cytotoxic cell in defence against viral infection (Appay et al., 2002), however the acquired immune system takes up to a week to develop (Parham, 2003). On the other hand the innate immune system is able to provide an immediate response to infection and thus is likely to be critical early in the infective process. NK cells are part of the innate immune system, and have a central role in early defence against infectious agents particularly viruses, and control of tumour growth. New evidence is emerging for a critical role played by NK cells, in the control of herpes virus infection, and this is discussed in more detail at the end of this section (See section 2.10.6 page 61).

2.10.1 Key characteristics of NK cells

Human NK cells comprise approximately 10% of the peripheral blood lymphocytes, and are also present in the lymph nodes, spleen, bone marrow and placenta. NK cells develop in the bone marrow from CD34+ progenitor cells. NK cells express CD56 and/or CD16 but are CD3/αβ TCR negative, they are thymus independent and do not undergo germ line rearrangements of their receptors (Farag et al., 2002). NK cells are cytotoxic cells and lyse target cells by the release of perforin which allows the subsequent traffic of cytotoxic granzymes to the target cell (Russell and Ley, 2002). In addition they can mediate antibody-dependent cellular cytotoxicity via CD16 (Fcg RIII), and are an important source of both antiviral and immunoregulatory cytokines.

NKT cells share some characteristics with both NK cells and T cells, they express NK cell receptors but also an invariant CD3/αβ TCR (Godfrey et al., 2000).

Recently much progress has been made in our understanding of the mechanisms that regulate NK cell activation and in particular recognition of virus infected and malignant cells. It is hoped this progress will eventually allow NK cells to be utilised in clinical immunotherapy of cancers and possibly infections (Farag et al., 2002).

2.10.2 NK cell subsets - $CD56^{bright}$ and $CD56^{dim}$ cells

In healthy adults, two distinct populations of NK cells have been identified by the density of cell surface expression of CD56 (Lanier et al., 1986), and these subsets

differ in their ability to produce cytokines and lyse cellular targets (Cooper et al., 2001a). Around 90% of resting NK cells are $CD56^{dim}$, and this subset is cytotoxic and expresses high levels of CD16. The remaining 10% of cells are $CD56^{bright}$ and resting $CD56^{bright}$ cells show less cytotoxic activity than the $CD56^{dim}$ subset. However, after stimulation with IL 2, $CD56^{bright}$ cells produce high levels of immunoregulatory cytokines, and also show enhanced cytotoxic activity (Cooper et al., 2001b; Cooper et al., 2001a). At present it is not clear whether the two subsets represent different stages of NK development or have different roles in the pathogenesis of human disease.

2.10.3 NK cell function is controlled by opposing signals.

In 1986 Karre and colleagues predicated that NK cells recognize and target autologous cells, which have either down regulated or altered major histocompatibility complex (MHC) class 1 expression (Karre et al., 1986). This was known as the "missing self" hypothesis. Since then it has become clear that NK cells express a number of both inhibitory and activating receptors which operate in concert to control NK cell function (Figure 2-2 page 56). Broadly speaking NK cells do not kill cells which express a full set of autologous MHC 1 molecules, but may be able to lyse allogeneic cells which express certain combinations of MHC1 (Valiante et al., 1997). NK cell receptor biology is a rapidly advancing field, and the nomenclature is complex; receptors can be classified both by their function; inhibitory or activating, and by their structural characteristics. As our knowledge grows it is evident that the expression of a number of these receptors is promiscuous with much overlap in expression of receptors between NK, NKT and CD8+T cells. As there are marked differences between the nomenclature and function of the mouse and human NK cell receptor systems, only the human system will be discussed.

Three major groups of receptors are recognized based on their structural characteristics, Killer Immunoglobulin Receptors (KIRs), Natural Cytotoxicity Receptors (NCR), and the C-type Lectin Domain family (Moretta et al., 2000; Moretta et al., 2002; Lanier, 2001). KIRSsbelong to the immunoglobulin superfamily and recognize different groups of MHC including MHC-A, MHC-B and MHC-C. The NKG2 family is characterized by a C-type lectin domain, and is a heterodimer of

a common subunit, CD94, and a unique unit encoded by the NKG2 family. The NCRs also belong to the immunoglobulin gene superfamily but have little homology with known human cell surface molecules. The KIR family and the CD94/NKG2 family include both inhibitory and excitatory receptors, whereas currently all members of the NCR family identified are activating type receptors.

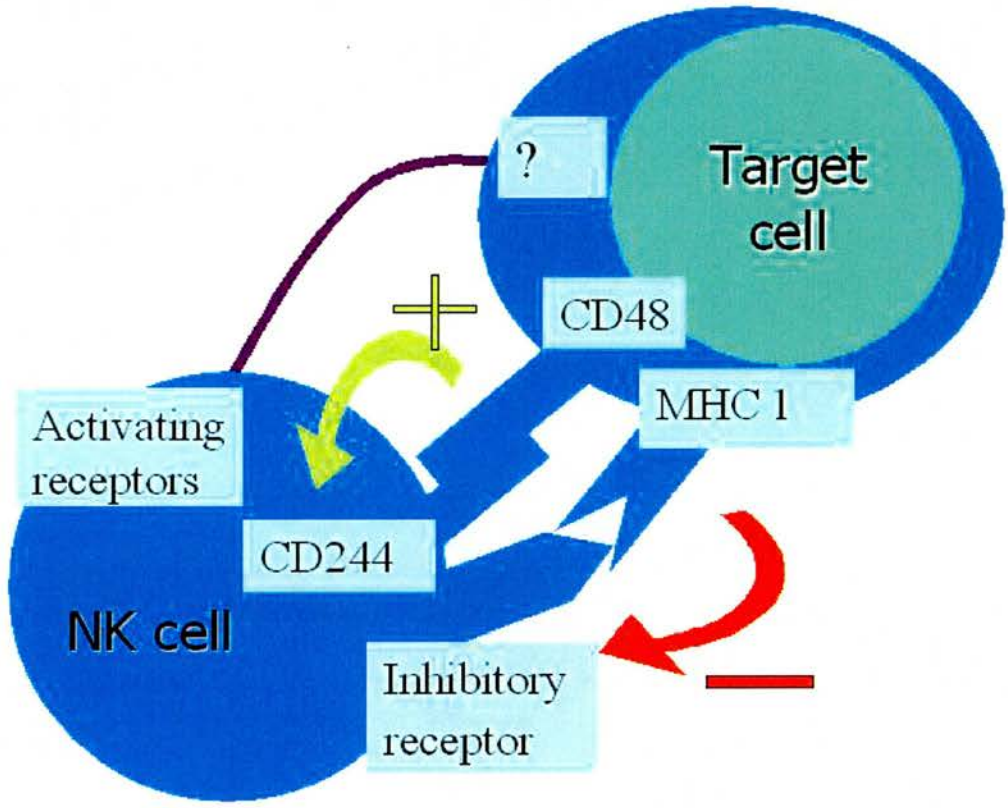


Figure 2-2 NK cell activation is controlled by inhibitory and activating receptors

2.10.3.1 Inhibitory Receptors

NK cells express a series of cell surface inhibitory receptors, which recognize MHC 1 expression on the cell surface of autologous cells. This recognition of normal MHC 1 on healthy cells is believed to protect against damage to self. On the other hand alteration of MHC 1, either on virus infected or malignant cells precludes this inhibitory interaction, allowing activating receptors to dominate, and trigger NK cell mediated lyses. For example, NK cells are able to lyse a MHC 1 negative LCL

known as 721-221 (Shimizu et al., 1988). However, transfection of MHC 1 genes into a previously NK cell susceptible target inhibits NK cell killing (Litwin et al., 1993).

At least 3 groups of inhibitory receptors have been identified in humans, KIRs, members of the NKG2A/CD94 family, and CD85j and CD85d based motifs (Lanier, 1998) (Table 2-9 page 57). The KIR group of receptors recognizes different alleles of MHC-A, MHC-B, or MHC-C molecules. Each type of KIR, is expressed by only a subgroup of NK cells, though all mature NK cells will express at least one receptor for self MHC 1, and this allows recognition of even minor changes in self MHC. KIR expression is not limited to NK cells, but also occurs on subsets of activated CD8+ αβTCR+ T cells (Mingari et al., 1998). NKG2A/CD94 also recognizes MHC molecules, but class E (Lanier, 2001).

Table 2-9 Inhibitory NK cell receptors

Inhibitory receptors	Family	Ligands
KIR2DL	KIR	MHC-C
KIR3DL	KIR	MHC-B MHC-A
CD94/NKG2A (CD159a)	C type lectin domain	MHC-E
CD85j, CD85d	C type lectin domain	MHC class I

2.10.3.2 Excitatory receptors

NKG2D, is an activating NK receptor, which is part of the C type lectin domain family and again its expression is not limited to NK cells but it is present on χδTCR+T cells and CD8+ αβTCR+ T cells (Bauer et al., 1999). A number of ligands have been recently recognized including the human MHC, MHC– class–I-chain related A and B antigens, (MICA and MICB), and UL-16 Binding Proteins (ULBPs). The MICA antigens have been the focus of much interest as they are expressed at low levels in healthy tissues, but are frequently over expressed in tumours, and virally infected tissues (Jamieson et al., 2002).

The NCR family of receptors includes, NKp46, NKp30 and NKp44 (Moretta et al., 2000). NKp46 was the first to be identified, and cross linking of the receptor leads to calcium mobilization, target cell cytotoxicity and cytokine release (Sivori et al., 1997; Sivori et al., 1999; Pessino et al., 1998). Interestingly, NKp46 expression appears to be limited to NK cells, and expression is high in the majority of donors (Moretta et al., 2000), so the receptor may prove to be a useful means of identifying a pure NK cell population. NKp30 is also expressed by all NK cells, however is thought to co-operate with both NKp46 and NKp44 in the lysis of target cell lines (Pende et al., 1999). NKp44 is not expressed by resting cells but is expressed in vitro following NK cell activation with IL-2, and again is thought to cooperate with NKp46 in the lysis of target cell lines (Vitale et al., 1998). Inhibition of NKp46 activity by a blocking antibody has reduced the lysis of a number of cell lines including EBV transformed LCL and tumour lines (Parolini et al., 2000), however the targets ligands for the NCR receptors have yet to be identified.

Working in concert with the NCRs are a number of co-receptors, which are structurally different, but in vitro studies suggest cooperation is required with the NCRs to signal cell activation. CD244/2B4, and NTB-A, are included in this group; CD244 is only able to trigger activation on cells with a high surface density of NKp46 and requires simultaneous engagement of NKp46 for activation to occur. For example, NK cell clones with high surface density of NKp46 were triggered by engagement of CD244 by antibodies, whereas NK cells with low cell surface expression of NKp46 were not (Sivori et al., 2000). As previously discussed CD244 may be important in the recognition of EBV infected cells as it is able to ligate CD48 and of note signals via SAP (Figure 2-2 page 56).

The KIR group of receptors is encoded on chromosome 19, by a family of genes known as the leukocyte receptor complex. Interestingly, the KIR genes are very polymorphic; within a population individuals have highly diverse NK cell repertoires and unless related almost always have different KIR types (Uhrberg et al., 1997). In addition the KIR group of genes appear to be rapidly evolving. Substantial differences were found between human and chimpanzees KIRs despite the fact that we diverged from chimpanzees 5 million years ago (Khakoo et al., 2000), a relatively

short time in evolutionary terms. Of note, the genes encoding CD94 and NKG2 do not show the same degree of genetic diversity (Shum et al., 2002).

Table 2-10 Activating NK cell receptors (Adapted from Cerwenka and Lanier, 2001).

Activating Receptors	Family	Ligands
NKp46	Natural cytotoxicity receptors (NCR)	Unknown
NKp30	NCR	Unknown
NKp44	NCR	Unknown
CD244/2B4	NCR co-receptor	CD48
NTBA	NCR co-receptor	Unknown
CD16	immunoglobulin	IgG
KIR2DS	Killer immunoglobulin-like receptor (KIR)	MHC-C
NKG2D	C type lectin domain	MIC, ULBP

MIC-MHC-class-1-chain-related molecules. ULBP-UL16-binding protein.

2.10.4 Cytokines acting on NK cells

As explained, the outcome of NK cell interaction with a possible target cell is the result of a concert of signals from activating and inhibitory receptors. How this is influenced by other mechanisms such as chemokine and cytokine networks is not yet clear. However, NK cells express cytokine receptors and are themselves an important source of immunoregulatory cytokines. At present, most of our knowledge of NK cell cytokine networks derives from in vitro tissue culture systems.

NK cells are the target of cytokines and monokines which are expressed by bystander cells following viral infection, in particular α/β interferons are released following viral infection and are potent inducers of NK cell mediated cytotoxicity (Cerwenka

and Lanier, 2001; Orange and Biron, 1996; Nguyen et al., 2002). NK cells also express receptors for monocyte-derived cytokines (monokines) including IL-1, IL-10, IL-12, IL-15 and IL-18 (reviewed by Biron et al., 1999). IL-12 and Tumour Necrosis Factor (TNF) are both able to stimulate the production of INF γ by NK cells (Tripp et al., 1993; Hunter et al., 1994; Paya et al., 1988; Paya et al., 1988).

The response of $CD56^{bright}$ and $CD56^{dim}$ cell subsets to IL-2 in vitro has been studied in detail (Cooper et al., 2001a). All NK cells express a functional heterodimeric IL-2 receptor with intermediate affinity for IL-2, however it is the $CD56^{bright}$ subset which has constitutive expression of a high affinity IL-2 receptor, and a high proliferative response to low dose IL-2 in vitro. In contrast, $CD56^{dim}$ cells have a very limited proliferative response to IL-2 (Cooper et al., 2001b). Other differences between the bright and dim subsets have been identified, including the expression of a functional CC-chemokine receptor 7(CCR7), and expression of high levels of functional L-selectin- adhesion molecule on the $CD56^{bright}$ cell subset (Kim et al., 1999). Both of these receptors are involved in cell trafficking and it has been suggested that $CD56^{bright}$ cells are able to traffic to secondary lymphoid organs. Indeed $CD56^{bright}$ cells have recently been identified as the dominant NK cell in human lymph nodes, and may provide an important point of contact with the acquired immune system (Fehniger et al., 2002).

The end result of NK cell activation is initiation of the cells effector functions. However, we are now beginning to recognize that NK cells may be 'primed' before activation occurs, indeed initially the cells must migrate to the site of the target, be it infection or malignancy. Our knowledge of NK cell priming and migration is limited; recent studies have suggested that bi-directional cross talk between dendritic cells and NK cells may be important (Piccioli et al., 2002). In addition, co-stimulatory molecules, engaged by NK cells (such as intracellular adhesion molecules or CD58) alongside the cytokine networks discussed above, may have a role in cell priming before cell activation occurs (Zitvogel, 2002).

2.10.5 NK cells are a source of immunoregulatory cytokines

NK cells are known to produce a number of pro inflammatory cytokines, including IFN γ, TNF β, granulocyte/macrophage colony stimulation factor and beta

chemokines, macrophage inflammatory protein 1α and macrophage inflammatory protein 1β (Orange and Biron, 1996). Again differences have been identified between the $CD56^{bright}$ cell and $CD56^{dim}$ cell subsets. In studies on human NK cells, the $CD56^{bright}$ cell population, either stimulated with monokines or phorbal 12-myristate 13-acetate (PMA) and ionomycin, are the major source of the cytokines detailed above (Cooper et al., 2001b).

2.10.6 NK cells and control of viral infection

The strongest evidence for the role of NK cells in control of herpes virus infections remains the case histories of individuals with NK cell defects who have developed recurrent herpes virus infections (Biron et al., 1989 and reviewed in Orange, 2002). Isolated NK cell defects are rare, but defective NK cell responses have also been documented in several other syndromes in which severe EBV infection occurs, these include CAEBV (Joncas et al., 1989), EBV linked viral haemaphagocytic syndrome (Kogawa et al., 2002) and Chediak–Higashi syndrome (Merino et al., 1983). Chediak –Hiagashi is a rare autosomal recessive lysosomal storage disorder in which hypopigmentation and NK cell defects occur. The important role of NK cells in the pathogenesis of XLP has been discussed (see section 2.7.8.2 page 47).

Earlier studies on the immune response to EBV have identified raised NK cell numbers during IM (Tomkinson et al., 1989). Previous investigators have also documented a decreased ability of ex vivo PBMC from IM patients to kill the NK target cell line K562 (Williams et al., 1989). However, when this work was carried out NK cell separation methods were limited. Since then, much progress has been made in our ability to both identify NK cells in the peripheral blood, and isolate them from the PBMC population either by use of magnetic beads or FACS methodology.

Recently, it has become clear that certain NK receptors are able to directly recognise viral antigens on infected cells. For example, NK cells expressing NKp46, one of the major human activating receptors, bind to and lyse cells expressing the haemagglutinins of both influenza and parainfluenza viruses (Mandelboim et al., 2001). Furthermore, susceptibility to mouse cytomegalovirus has been linked to deletion of an activating NK cell receptor (Daniels et al., 2001; Brown et al., 2001; Lee et al., 2001), which recognises the viral protein M157 (Arase et al., 2002).

Interestingly, Lanier has noted that individuals with NK cell defects do not appear to be at risk of severe influenzae infection, therefore the relevance of the interaction between Nkp46 and haemagglutinins to the control of influenzae infection in humans remains unclear (Cerwenka and Lanier, 2001).

Viral infection may lead to reduced expression of MHC 1 on the surface of a cell, and this is thought to be associated with evasion of CTL recognition by the virus. On the other hand, this is likely to render the cell susceptible to recognition by NK cells. However a number of viruses also have mechanisms which may allow them to escape detection by NK cells (Farrell et al., 1997, and reviewed in Orange et al., 2002). For example, human CMV encodes a class 1 homologue on the cell surface, which is thought to block NK cell activity by ligation of inhibitory NK cell receptors (Beck and Barrell, 1988). In addition, in mice, the CMV M152 encoded gp40 glycoprotein has been shown to downregulate ligands for NKG2D (Lodoen et al., 2003). Following human CMV infection, intracellular sequestration of ligands for NKG2D occurs, again allowing the virus to evade detection by NK cells (Dunn et al., 2003). Other mechanisms of viral evasion of NK cells are well recognized, such as virus encoded cytokine binding proteins (Orange et al., 2002), which would inhibit NK cell recruitment and activation. At present it is not known if EBV utilities any of these mechanisms.

Following the unraveling of the interaction between NK cells and mouse CMV infection, Lanier and Arase have suggested that a key evolutionary pressure on NK receptor development may be viral infection (Arase and Lanier, 2002).

2.11 Varicella Zoster Virus

Varicella zoster virus (VZV) is an alpha herpes virus, which is solely a human pathogen. Unlike the other herpes viruses, primary infection in childhood is normally symptomatic, (chicken pox), although only mild illness occurs in the majority of cases. Primary infection in adults, tends to be much more severe, particularly in pregnancy. After primary infection, the virus establishes latency in sensory nerve ganglia. Reactivation of the virus is common, particularly in the elderly and causes

Herpes Zoster (shingles). Host immunosuppression can lead to both severe primary infection and life threatening systemic disease associated with reactivation.

2.11.1 Primary infection- Chickenpox

Primary VZV infection, or chicken pox, is normally a mild illness characterised by a febrile illness and vesicular rash, and no significant complications. In temperate climates over 95% of the population is infected under the age of 10. The incubation period is 10-21 days, and the virus is highly infectious with 70-90% of seronegative household contacts contracting the virus (Arvin and Gershon, 1996).

Antiviral therapy is appropriate in severe cases, though should ideally be used within 24 hours of the onset of disease. Aciclovir and the newer pro drugs such as valaciclovir, inhibit the viral DNA polymerase; they are selective for the virus as only the viral thymidine kinase phosphorylates and activates the drugs.

2.11.1.1 Immune response to primary VZV infection

As demonstrated by the severity of infection in children with defects in cellular immunity, the T cell response is critical to the control of primary VZV infection. In comparison, those with B cell defects appear to be at less risk of disseminated disease (Cohen et al., 1999).

Studies of *ex-vivo* T cells from individuals with primary VZV have enabled the identification of a number of characteristics of the cell mediated response. As in primary EBV infection, T cell populations are activated and express increased levels MHC-2 (Arvin, 1992). Strangely, there appears to be a prolonged delay in the development of VZV specific T cells, as they are not easily detected until 24-72 hours after the appearance of the rash. However, the majority of this work was done before the advent of more sensitive techniques for detection of antigen specific cells (such as tetramers) and use of these technologies may allow identification of VZV specific cells earlier in the disease course. Both virus specific CD4+ and CD8+ cell subsets have been identified during primary infection. The CD4+ cells are mainly of a TH1 phenotype and synthesis IL-12 and IFN-γ (Asanuma et al., 2000). At present

there is no animal model of primary VZV infection in which to investigate the pathogenesis further.

2.11.2 Live attenuated VZV vaccination

VZV was the first herpes virus for which a vaccine was available and widely used. The vaccine is a live attenuated virus, based on the OKA strain. The vaccine was licensed in the USA in 1995 (1996), and it is more widely used in the USA than the UK. It is 85-90% effective in preventing primary infection, and in those who do go on to develop chickenpox, the condition is usually mild (Wise et al., 2000). It may be particularly useful in seronegative health care workers. Unfortunately a live attenuated vaccine is not suitable for either pregnant women or the immunosuppressed, both high risk groups for severe VZV infection. However, the vaccine is indicated for leukaemia patients in remission, who seem to be especially vulnerable to severe infection.

3 Materials and Methods

3.1 Addresses for suppliers

Applied Biosytems	Applied Biosytems 850 Lincoln Centre Drive Foster City, CA 94404
BD Biosciences	10975 Torreyana Road San Diego, CA 92121-1106
DAKO	DAKO Corporation 6392 Via Real CA 93013 USA
Diagnostic Scotland	Law Hospital Carluke Lanarkshire Scotland ML8 5QZ
European Collection of Cell Cultures	CAMR Salisbury Wiltshire SP4 OJG
Exalpha Biologicals	20 Hampden Street Boston MA 02119
Fischer Scientific	Fisher Scientific UK Ltd Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG
Fred Baker Scientific	3 Lancer court Astmoor Industrial Estate, Runcorn, Cheshire WA7 1PN
Immunotech	Immunotech, Coulter, 13276 Marseille Cedex 9 France
Insight Biotechnology	P O Box 520, Wembley, Middlesex, HA9 7YN, UK, Tel: +44 20 8385 0303
Microgen Bioproducts	1 Admiralty Way, Camberley, Surrey, GU15 3DT,
Miltenyi Biotech	Miltenyi Biotech Friedrich-Ebert-Str 68 51429 Bergisch Germany
MWG value read	www.mwgbiotech.com
Molecular Probes	Molecular Probes Inc 4849 Pitchford avenue Eugen Or 97402-9165, USA
Perkin Elmer	45 William Street Wellesley, MA, 02481-4078, USA
Qiagen	Boundary Court, Gatwick Rd, Crawley, RH10 9AX
Santa Cruz Biotechnology	2161 Delaware Avenue, Santa Cruz,

	California 95060
Sarstedt	68 Boston Rd, Leicester LE4 1AW
Scientific Laboratory Supplies (SLS)	Unit 17, Coatbridge Business Centre, 204 Main Street, Coatbridge, Lanarkshire ML5 3RB
Shandon Scientific Ltd	Chadwick Rd, Runcorn, Cheshire WA7 1PR
Sigma	Sigma-Aldrich Company ltd. Fancy Rd, Poole, Dorset, BH12 4QH
Sigma Genosys	www.genosys.co.uk
Vector	Vector Laboratories inc 30 Ingold Road Burlingame, CA 94010 USA.
Zymed	Zymed laboratories 561 Eccles Avenue So. San Francisco, CA 94080

3.2 Laboratory consumables, chemicals, and kits

All laboratory consumables were supplied by Fisher Scientific UK or Scientific Laboratory Supplies unless otherwise stated. All tissue culture consumables were supplied by Fred Baker UK. All laboratory chemicals were supplied by Sigma unless otherwise stated.

Amplitaq with 10X PCR buffer II & $MgCl_2$ Solution	Applied Biosytems
Big dye rapid reaction mix	Applied Biosytems
Animal Serum- mouse, rabbit, horse, pig	Diagnostic Scotland
Cell dissociation medium	Sigma
Cytospin filters and cartridges	Shandon
DAKO fluorescent Mounting Medium	DAKO
DAKO antibody diluent with background reducing components	DAKO
DAKO ISH kit and EBERS probe	DAKO
DAKO Liquid DAB+ Substrate-Chromagen Solution	DAKO
DAKO Protein Block Serum Free	DAKO

DAKO Ultramount aqueous permanent Mounting Medium, Ready to Use	DAKO
ImmunoEdge Pen	Vector
Infectious Mononucleosis absorption kit (monospot)	Microgen Bioproducts
Invitrogen Easy DNA kit	Invitrogen
Haematoxylin Nuclear Counter Stain	Vector
MACS NK Cell Isolation Kit	Miltenyi Biotech
MACS Separation Columns MS+ Columns	Miltenyi Biotech
MACS Magnet cell separator	Miltenyi Biotech
Methyl Green Counter stain	Vector
Perm/Wash Buffer	BD Pharmingen
Primers for XLP PCR and EBV PCR	Sigma genosys
Qiagen quick gel extraction kit	Qiagen
Starstedt vacutainer system for peripheral blood collection.	Starstedt
StrepABComplex/HRP	DAKO
Sterptavidin/Biotin Blocking Kit	Vector
Taq polymerase	PE biosytems
TOPRO 3	Molecular Probes
Vectastain ABC-AP Kit	Vector
Vector Blue Alkaline Phosphatase Substrate Kit	Vector
Vectastain ABC Kit	Vector
VectorMount Mounting Medium	Vector
Vector Red Alkaline Phosphatase	Vector
Avidin Biotin Blocking Kit	Vector

3.3 Antibodies

Table 3-1 Details of antibodies used

Target	Isotype/ species	Label	Use	Dilution	Storage	Source
CD244/ 2B4	Mouse IgG_1	PE	Flow	10ul per sample	4^0C	Immunotech
EBV LMP1	Mouse IgG_1	-	IHC	1:25	4^0C	DAKO
EBNA-3B	Mouse IgG_1	-	IHC	1:25	4^0C	Exalpha Biologicals
EBNA 3a	Mouse monoclonal	-	IHC	1:20	4^0C	Kind gift from Prof Rowe Cardiff
EBNA 3c	Mouse Monoclonal	-	IHC	1:20	4^0C	Kind gift Prof Rowe Cardiff
INF- γ	Mouse IgG_1	-	IHC & Flow	1:30	4^0C	BD Pharmingen
CD56	Mouse IgG_1	-	IHC	1:30	4^0C	Zymed Laboratories
CD3	Mouse IgG_1	-	IHC	1:50	4^0C	DAKO
Goat IgG	Horse	Biotin	IHC	1:500	4^0C	Vector
SAP	Rabbit	-	IHC	1:20	4^0C	Santa Cruz Diagnostics

SAP	Goat	-	IHC	1:20	4^0C	Santa Cruz Diagnostics
Mouse IgG	Goat	Biotin	IHC	1:500	4^0C	Vector
Rabbit IgG	Goat	Biotin	IHC	1:500	4^0C	Vector
CD56	Mouse IgG_1	PE	Flow	10ul per sample	4^0C	BD Pharmingen
CD16	Mouse IgG_1	FITC	Flow	10ul per sample	4^0C	BD Pharmingen
CD8	Mouse IgG_1	FITC & PE	Flow	10ul per sample	4^0C	BD Pharmingen
CD3	Mouse IgG_1	Cy-Chrome / FITC & PE	Flow	10ul per sample	4^0C	BD Pharmingen
CD19	Mouse IgG_1	FITC	Flow	10ul per sample	4^0C	BD Pharmingen
CD4	Mouse IgG_1	FITC & PE	Flow	10ul per sample	4^0C	BD Pharmingen
SLAM	Mouse IgG_1	FITC	Flow	5cg per sample	4^0C and -20^0C long term	Sheffield University Hybridoma
Anti Goat IgG	Rabbit	Alexa Flour^R	Flow and IHC	1:500	-20^0C	Molecular Probes
To Pro 1 iodide	-	-	Flow Nucleic acid stain	1:10 and 10ul per tube	-20^0C	Molecular Probes

IHC- immunohistochemistry, Flow- flow cytometry- no conjugate, FITC Fluorescein-5-isothiocyanate, PE- R-phycoerythrin

3.4 Materials and Solutions

Citrate Buffer

Stock A Citrate Buffer	Stock B Citrate Buffer	Working solution 10 mM Citrate Buffer
4.2 g Citric Acid	14.7 g Sodium Citrate	18 ml Stock A
20 mls ddH_20	500 mls ddH_20	82 ml Stock B
		Fill to 1 litre with ddH_20 and pH to 6.0

Phosphate Buffered Saline (PBS)

NaCl	0.8% w/v
KCl	0.02%w/v
Na_2HPO_4	0.02%w
KH_2PO_4	0.15%w/v

Tris Buffered saline (TBS)

(made up in BS)

Tris HCL pH 8.0	10mM

Standard Tissue Culture Medium (RPMI) store at 4^0C, and use within 28 days

L-glutamine	2mM
Penicillin	100IU/ml

Streptomycin	100ug/ml
Fetal calf serum	10%v/v
Made up in 1x RPMI 1640.	

Freezing medium store at 4^0C, and use within 28 days

Fetal Calf Serum	90% v/v
DMSO	10% v/v

DNAase store at 4^0C, and use within 2 days

Fetal Calf Serum	70%v/v
Heparin	100 units per ml
$MgSO_4$	15mM
DNAase	2000 units per ml

Flow cytometry buffer, store at 4^0C, and use within 28 days

Bovine Serum Albumin	1% w/v
Sodium azide	0.1%w/v
EDTA	0.02%v/v
PBS	Made up in PBS

MACS Buffer, store at 4^0C, and use within 24 hours

Bovine Serum Albumin	1% w/v
EDTA	0.02%v/v
PBS	Made up in PBS

10 X PCR Buffer 1 (autoclave before use as 10 x PCR buffer), store at room temperature

Tris-HCL (ph 8.8)	670 mM
(NH4)2 SO4 (enzyme grade)	166 mM
$MgCl_2$	67 mM

1x Tris EDTA T(0.1)E, store at room temperature

Tris HCL(ph 8.00)	10mM
EDTA	1mM

TCR-T.0.1.E with Creole red, autoclaved before use (28g sucrose and 8mg Cresol red) in 100ml T.O.1.E

3.5 Cell Lines

Cell line	Source	Medium	Reference
B958	LCL Marmoset	Standard tissue culture medium	(Miller et al., 1972)
K562	ECACC	Standard tissue culture medium	(Klein et al., 1976)
721.221	Imperial College London K.Eleme	Standard tissue culture medium+ 10%v/v Amino acids	(Shimizu et al., 1988)

3.6 PCR and sequencing techniques

3.6.1 DNA extraction

Tissue specimens were snap frozen and stored at -70^0 C. Samples (3.5-100mg) were thawed on ice and pulverised with a pellet mixer in 200 ul of PBS. The Invitrogen Easy DNA kit was used, as per manufacturers instructions. 350 ul of solution A was added to the cell suspension and the mixture was vortexed until evenly dispersed.

The cell suspension was then incubated at 65^0 C for 10 minutes and briefly centrifuged. 500ul of chloroform was added and again the tube vortexed until the viscosity decreased and the mixture was homogenous. The suspension was then centrifuged at 13,000rpm for 15 minutes at 4^0 C; two separate layers form, and the upper layer was transferred into a clean microfuge tube. The DNA was then precipitated by addition of 1ml of 100% alcohol, and freezing overnight at -20^0 C. The DNA was then centrifuged at 13,000rpm for 15 minutes at 4^0 C, the ethanol removed and the pellet allowed to air dry. The pellet was then resuspended in molecular grade water, and stored at 4^0 C (short term) and -20^0 C (long term).

3.6.2 PCR reaction

Two PCR methods were used, the first followed the method of (Coffey et al., 1998) the second was adapted from (Yin et al., 1999). A Biometra T3 thermocycler PCR machine was used throughout.

Method 1	1 sample (ul)
Buffer	1.5
dNTPs	1.5
BSA	0.495
BME	0.21
Taq polymerase	0.18
TCR	3.2
Primer *forward* (100ng/ul)	1.5
Primer *reverse* (100ng/ul)	1.5
DNA(10ng/ul)	5
Made up in ultrapure water	Total 50ul

Method 2	1 sample (ul)
Buffer (15mM Mg)	5

DNTP (20mM)	0.5
Primer *forward* 0.1nM/ul	0.5
Primer *reverse* 0.1nM/ul	0.5
Taq polymerase	0.4
DNA (50ng)	4
Made up in ultrapure water	Total 50ul

3.6.3 Cycling conditions

The cycling conditions were the same for both methods. The reaction started with 5 minutes denaturing at 94^0 C, followed by 35 cycles of three steps; 93^0 C for 30 seconds, primer specific annealing temperature for 30 seconds, and 72^0 C for 30 seconds. This was followed by an extension step of 5 minutes at 72^0 C.

3.6.4 Primer selection

The oligonucleotide primers used for the PCR were designed from the published sequences for all 4 exons, and were chosen to amplify the whole coding exon. The primer sequences were situated just outside the coding region for each exon (Coffey et al., 1998). (See Table 2-1 page 18).

Table 3-2 Primers used for PCR and sequencing SH2DIA/SAP

Exon		Primer sequence (5'-3')	Anneal temp ^{0}C	Size (bp)
Exon 1 first round	F	GTTGAGCTAAGTTATTCCTG	60	729
	R	TGAGGCGAAAGTGTGTTCCA	60	
Exon 1 second round	F	ACAGAAGCATTACTAAGC	60	568
	R	CCAGCTGCTGTTGCCCAC		
Exon 2	F	CAATGACACCATATACGTGT	60	341
	R	GCTTTCTTAATGATCCATGA		
Exon 3	F	CAAGTTACACAAATGTTTA	55	308

	R	CTTGGACTCATAACTCTCTG		
Exon 4	F	TCATTGTGAGTTTTATGCAG	55	233
	R	GCTCACCGAACTGTATTA		

3.6.5 Control Template

A positive and negative control was used with each PCR reaction, genomic DNA was used as a positive control and molecular grade water was used to replace the DNA as a negative control.

3.6.6 Gel separation

PCR products were separated on either 2.5 % or 4% agarose and visualised by ethidium bromide (EB) staining; 2.5 ul of EB per 50 ml gel or buffer. TBE buffer was used in the gel tank. The gels were run at 120 V for 40-80 minutes. A 1 Kb DNA marker was used.

3.6.7 Gel products – cleaned before sequencing.

The PCR products were purified directly after separation on a gel. QIAGEN QIAquick Gel Extraction Kit was used; instructions and reagents were supplied by the company. Appropriate bands were cut from the agarose gel using a minimal amount of UV exposure. The gel band was weighed, and 6 volumes of buffer QC were added to the gel. (The volumes was calculated using the principal of 100mg=100ul). The gel and buffer were incubated at 50^0C for 10 minutes, with vortexing every 2 to 3 minutes. When the gel had dissolved completely 1 volume of isopropranol was added to the mixture and mixed gently. The solution was applied to a spin column, and centrifuged for 1 minute. The column was washed with 500ul of Buffer QC, to remove excess agarose. 750 ul of Buffer PE was added and the column centrifuged for 1 minute, twice. The DNA was eluted with the addition of 30 ul of elution buffer (10 mM Tris-Cl, ph 8.5), and the product stored at 4^0C.

3.6.8 Sequencing and ethanol precipitation

Sequencing was carried out using BIG DYE Terminator Ready Reaction kit. In brief 8ul of clean PCR product was mixed on ice with 2ul of primer (concentration of

1.6pmole/ul), 8ul of Big Dye and 4ul of water. The reaction was 25 cycles of 95^0C for 30 seconds, 50^0C for 20 seconds, and 60^0C for 4 minutes, followed by a holding step at 4^0C.

A second sequencing service was also used, the commercial service MWG Value Read. Clean PCR products were sent at a concentration of DNA = 20ng/100 nucleotides.

PCR and sequencing products were ethanol precipitated prior to use. Either 10ul purified PCR product (for MWG sequencing) or 10ul of the sequencing reaction were mixed with 1ul of 3M NaOAc and 200 ul of 100% Ethanol, at -20^0C, vortexed and left on ice for 20 minutes. The tube was then centrifuged at 15000rpm for 30 minutes at 4 ^{0}C, and then washed (once or twice) with 70% alcohol, and again centrifuged at 15000rpm for 15 minutes.

3.6.9 BLAST search

Sequences were entered into the National Centre for Biotechnology (NCBI) BLAST (standard nucleotide search engine http://www.ncbi.nlm.nih.gov/blast/) and sequences were compared with the wild type sequence for the 4 XLP exons. The full sequence is available on European Molecular Biology Laboratory (www-db, embl-heidelberg.de) (EMBL) sequence data base (ID HSDSHP, accession number AL 023657), and this was used to verify the location of the sequence within the 4 exons.

3.7 Cell culture techniques

3.7.1 FICOLL- HYPAQUE Separation of PBMCs

50 ml Falcon tubes were filled with a volume of Ficoll equal to the volume of blood to be separated. Using a sterile pipette an equal volume of blood was over-layed on the Ficoll. The tubes were centrifuged at 2200 rpm at 20°C for 20 minutes. The plasma was collected using a Pasteur pipette and stored at -20°C. A sterile pasteur pipette was used to collect the mononuclear cell layer which were transferred to a clean centrifuge tube. The tube was filled with HBSS and centrifuged at 1200 rpm (330g) at 20°C for 7 minutes. The supernatant was discarded and the pellet

resuspended in HBSS. The viable cells were counted using the trypan blue exclusion test and a haemacytometer.

3.7.2 Cell culture

All cells were cultured in an humidified 5% CO_2 incubator at 37 ^{0} C, and fed as required, usually every 2-3 days with the appropriate tissue culture medium. Adherent cell lines were split once the growth was 70-80% confluent; cell dissociation medium and cell scrapers were used to remove adherent cells and the cells were washed in tissue culture medium prior to resuspension. Cells grown in suspension were split when the tissue culture flask was approximately one third full.

3.7.3 Cell wash

All cell washes were carried out in HBSS or RPMI at 1000rpm for 5 minutes, in a bench top centrifuge.

3.7.4 Cell counts

A 1 in 1 dilution of cell suspension was made by adding 10µl of cells to 10µl of trypan blue. 10µl of stained cells was applied to the haemocytometer. The number of unstained cells (viable) in the 25 central squares was counted and divided by 5. This number, divided by 10 is equal to cell count per ml of cell suspension.

3.7.5 Viable storage of cells

Cell lines were fed the day before freezing. Cells were counted and spun in a centrifuge in culture medium at 1000 rpm (330g) for 5 min. Cells were resuspended in freezing medium at $5x10^6$ to $5x10^7$ cells per ml, and 1 ml was placed in a cyrovial. Vials were frozen for 24-48 hours at -70°C, followed by storage in liquid nitrogen

Cryovials of cells were thawed at 37^{O}C in a water bath for 1-2 minutes. Cells were immediately transferred into a Falcon tube containing 10-30 ml of warm HBSS. The tube was subsequently centrifuged at 1000 rpm (330g) for 5 minutes. The cells were then counted and used as required.

3.7.6 B958 virus preparation and titration

B958 cells were grown to 90% capacity in six 250 ml flasks, and then left covered in parafilm without feeding for 10 days. The culture was then spun in a centrifuge at 1200rpm for 5 minutes at 4 °C, the supernatant was then filtered through 0.8 μm filter and the cell pellet discarded. The supernatant (in Beckmann bottles) was then spun in a centrifuge at 18000rpm for 2.5 hours at 4 °C. The cell pellets were then resuspended in 1 ml of tissue culture medium, and 100μl was stored and frozen at -70°C in cryovials.

The virus was titrated by mixing EBV negative PBMC with a dilution series of virus stock ranging from neat virus, through $10^{-1,}$ $10^{-2,}$ $10^{-3,}$ $10^{-4,}$ $10^{-5,}$ $10^{-6,}$ to no virus. The cells were incubated in a 96 well plate (5 wells of each dilution , for 4 weeks with weekly feeding. At the end of four weeks the wells were scored for outgrowth under an inverted microscope. The virus titre equalled the dilution showing 50% growth.

3.7.7 Generation of PHA blasts

PBMC were suspended at 10^6 cells/ml in standard tissue culture medium, and 20μl of PHA per ml of cell suspension was added. Cells were placed at 2ml per well in a 24 well plate, and incubated at 37°C.

3.7.8 Generation of lymphoblastoid cell lines

PBMC were suspended at 10^6 cells/ml in standard tissue culture medium with 100-200 ul of B95-8 virus and incubated for 10 minutes at 37°C. 0.5ml per well was added to a 24 well flat bottomed plate. The plate was incubated at 37°C and fed with standard tissue culture medium every 3-4 days. When growth was established wells were split, and transferred to a flask as required.

3.8 Flow Cytometry

3.8.1 Antibody titration

The anti SLAM (A12, IgG1) antibody was grown at the Sheffield University Hybridoma Unit, and labelled with FITC at Scottish Antibody Production Unit.

A series of titration experiments were undertaken to investigate the optimal amount of SLAM antibody for staining 0.5-1 x 10^6 cells for flow cytometry. PBMC from 5 different donors were stimulated with PHA, and subsequently stained with varying amounts of FITC labelled SLAM antibody.

In summary maximum SLAM expression occurred at 4-6 hours following PHA stimulation and 5ug of labelled antibody per 0.5-1.0 x 10^6 cells, gave adequate staining but minimal background. This amount of SLAM antibody was therefore used for the flow cytometry experiments. All other antibodies were used at concentrations recommended by the manufacturer.

Table 3-3 Titration of SLAM antibody

Control	Hours of stimulation with PHA	SLAM staining		Comments
		SLAM 0-5mcg per tube	SLAM 5-10mcg per tube	
1	4	increase staining from 0-5	stable staining from 5-10	
2	No activation	Low level expression		Increase of SLAM expression between 4 and 6 hours Results verified in repeat experiment
	4/6	Increase from 0-5	Stable staining from 5-10	
3	16	stable expression from 0-10		atypical data
4	No activation	Low level expression		Resting cells have low expression of SLAM, therefore no useful for titration experiments
5	4	Increase from 0-5	Increase in background staining >8	

6	0-6	SLAM at 5mcg	Expression maximal at 4 hours, stable at 6 hours.	Maximum expression occurs between 4-6 hours post PHA stimulation

3.8.2 Cell surface staining

0.25-0.5 x 10^6 cells were placed in plastic tubes, and spun for 5 minutes at 1000rpm. Appropriate quantities of stain were added to each tube (Table 3-1 Details of antibodies used page 68) and incubated at 4°C for 15-30 minutes. The cells were then washed in 1ml per tube, cold FACS buffer, and spun for 5 minutes at 1000rpm. The supernatant was aspirated and the cells resuspended in 0.25-0.5 ml of FACS buffer and stored at 4°C. Tubes were analysed within 24 hours. The stained cells were analysed on a FACScalibur (Becton Dickinson). Samples were analysed by either 2 or 3 colour staining, plus TO-PRO. Cells were gated on forward and side scatter to identify the lymphocyte population, and dead cells were identified by staining for TO-PRO 3. In addition, in some analysis cells were further gated into a CD3 positive and negative population. Unstained cells and cells stained with appropriate isotype controls were used as negative controls. The data were processed on CELLQuest program (Becton Dickinson).

3.8.3 Intracellular staining

SAP is an intracellular antigen, and therefore the cell membrane must be permeated prior to the antigen staining, and flow cytometric analysis. Intracellular flow cytometry is a well established technique; the cell is permeated using a chemical such as saponin, and then stained with the appropriate antibody, followed by 2-3 wash steps to remove excess antibody.

The technique was established for analysis of antigens for which there is a labelled monoclonal antibody such as INF γ, an intracellular cytokine expressed in PHA stimulated PBMC. However, when intracellular staining of SAP was carried out, using an unlabelled polyclonal antibody, extensive non specific staining occurred and acceptable results could not be obtained. A number of steps were undertaken to reduce the non specific staining. These included use of a number of different

fluorescent secondary antibodies, increased number of wash steps, use of acetone absorption of the primary antibody, and use of alternative methods of cell permeabilisation. Therefore immunohistochemistry was used to analyse expression of SAP.

3.9 Immunohistochemistry

3.9.1 Cytospins

Fresh PBMCs were suspended at 1 x 10^6 cells/ml in PBS, and stored at 4^0 C. The cytospin chambers, slides (pre-coated with polysine) and a filter card were loaded into the cytospin, and the filters were pre wetted with 50ul of PBS. 50ul of PBMC suspension was added to each cytospin chamber and spun at 150rpm for 3 minutes. The slides were then fixed in acetone (at 4^0 C) for 10 minutes, and stored at -20^0 C.

3.9.2 Removing paraffin wax

Paraffin embedded sections were immersed in xylene for 5 minutes, followed by 5 minutes in 100% ethanol. The sections are rehydrated by sequential immersion for 3 minutes in 90%, then 70% then 30% ethanol, diluted in water. Finally slides were washed in tap water.

3.9.3 Microwave Antigen Retrieval

Antigen retrieval was carried out after de-waxing. The slides were washed in running water and immersed in citrate buffer in a plastic container, suitable for use in a microwave. The slides in buffer were microwaved on high for 3 x 5 minutes, with top up of buffer as needed. The slides were then washed in running tap water, followed by PBS. It was found that use of citrate buffer and microwave heating gave improved antigen retrieval compared to other systems tested including commercial buffers. Antigen retrieval was not required for frozen sections.

3.9.4 Single colour staining using an avidin-biotin amplification system

Immunohistochemistry was carried out using an avidin-biotin amplification system. In brief an unlabelled primary antibody of choice is use to target the designated

antigen (e.g. SAP). This is followed by a biotinylated antibody against the species of the primary antibody, and then followed by peroxidase conjugated avidin antibody. Finally a peroxidase dependent colour substrate is added (eg DAB (3, 3' – diaminobenzidine). Alternatively an alkaline phosphotase substrate may be used.

3.9.4.1 Single colour biotin- avidin peroxidase DAB (3, 3'– diaminobenzidine)

Slides, either paraffin fixed tissue, frozen section or cytospins were rinsed with PBS for 5 minutes. The slides were then incubated with 0.5% hydrogen peroxide for 10 minutes followed by a PBS wash. (All PBS washes were for 2 x 5 minutes). Next, the slides were incubated with normal serum diluted 1:5 in PBS, determined by the species of the secondary antibody. The slides were then incubated with the primary antibody (Table 3-1 Details of antibodies used page 68) followed by a wash then the secondary antibody (biotinylated and diluted 1:500), followed by a further wash. All antibody dilutions were carried out in DAKO antibody diluent. The slides were subsequently incubated with the peroxidase substrate solution (for 30 minutes), followed by a wash followed by DAB solution for 5 minutes in the dark. The slides were counter stained with haematoxylin for 1-2 minutes, followed by clearing by a rinse in 95% alcohol, 100% alcohol, 95% xylene, and 100% xylene. The slides were mounted and covered with a glass cover slip.

3.9.5 Optimization and method for sequential staining techniques.

In order to identify the cell type and function of the cells which expressed SAP a number of methods of visualizing 2 antigens in the same cell type were assessed (Table 3.4 page 82). A combination of 2 fluorescent antibodies, or DAB (brown) staining combined with Vector red failed to give satisfactory results. The problems derived both from the background staining from the SAP polyclonal antibody and the need for a good colour contrast to allow visualization of dual labeled cells. It was found that a first step reaction using DAB, for an antigen which was expressed by a small proportion of cells, followed by Vector Blue worked well.

For the double staining procedure the slides were stained with DAB as above, and then incubated with avidin biotin block for 15 minutes are room temperature. The

procedure was then followed as for single staining, except alkaline phosphatase substrate solution was used, with Vector Blue substrate. Slides were not cleared in xylene, but fixed with DAKO ultramount, which does not require a cover slip. In all cases the primary and secondary antibodies were from different animal species.

Table 3-4 Optimal colour combinations for dual labelling

Primary Substrate	Secondary Substrate	Result
Fluorescent Green label (FITC)	Fluorescent red label (TRITC)	Poor, excess background
Fluorescent Red label (TRITC)	Fluorescent Green label (FITC)	Poor, excess background
Peroxidase (DAB) brown	Alkaline Phosphatase (VECTOR RED)	Poor colour contrast
Alkaline Phosphatase (VECTOR RED)	Peroxidase (DAB) brown	Poor colour contrast
Peroxidase (DAB) brown	Alkaline Phosphatase (VECTOR Blue)	Good colour contrast, optimal results using less common antigen with primary substrate.

3.9.6 Counting

200 cells were counted on each slide in random fields. SAP/INF γ dual positive slides were graded as high (4+ dual positive cells per hundred cells), low (2 or less dual positive cells per hundred cells), or negative (no dual positive cells).

All the figures of IHC are marked with the microscope magnification; as digital photography was used, some of the figures were enlarged digitally.

3.9.7 In situ hybridisation for RNA detection using EBER probe.

The working procedure was followed for the PNA ISH Detection Kit (DAKO). All glass ware and water were washed to inactivate Rnase and double distilled water was used throughout. The slides were immersed in 99% ethanol followed by 96% ethanol, for 2 times 3 minutes at each concentration. The slides were incubated with proteinase K (diluted 1:1000) for 10 minutes in a humid chamber at room temperature. The slides were then washed in pure water (2 x 3 minutes) and then dipped in 96% ethanol, and air dried. 1 drop of FITC-conjugated PNA probe was added to the slide, and the slides were placed in a humid chamber at 55^0 C for 90 minutes. The slides were then immersed in the stringent wash solution (preheated to 55^0C) and washed at 55^0C for 30 minutes, followed by a brief immersion in TBS (room temperature). The slides were then incubated with 2-3 drops of ANTI-FITC/AP for 30 minutes at room temperature, and washed in TBS (2 x 3minutes), followed by pure water (2 x 1 minute). Finally, the slides were incubated with substrate solution for 30-60 minutes at room temperature, and washed in tap water, and mounted.

3.10 NK cell separation

PBMC were separated from whole blood using Ficoll method as described (3.7.1page76). The MACS beads separation method was followed as per the manufacturers instructions. In brief, cells were suspended in fresh cold (4 °C) MACS buffer at total volume of 80ul per 10^7 total cells. 30ul of hapten antibody cocktail (containing anti: CD3, CD4, CD14, CD15, CD19, and CD36 antibodies) was added per 10^7 cells and incubated for 15 minutes at 4 °C. Cells were washed in MACS buffer, and re-suspended at 80ul per 10^7 total cells in MACS buffer and 30ul of anti-hapten antibody cocktail was added per 10^7 cells and incubated for 15 minutes at 4 °C. The cells were washed in MACS buffer, re-suspended in 500ul of buffer per 10^8 total cells, and applied to an LS+ column in the magnetic field of the Midi MACS magnet. The NK cells were eluted with 4 x 3ml of MACS buffer, and the retained cells were the non NK cell fraction.

The cell purity was checked by standard FACS analysis on 5 occasions, using antibodies to CD56 and CD3, and found to routinely contain less than 0.5% CD3 positive cells on all occasions. Between 5-30% of the cells did not stain with antibodies to either CD3 or CD56, and so were checked for expression of CD14, CD19, and CD4, all of which were negative. On direct microscopy significant numbers of red blood cells were noted, and therefore represented the unstained cells in the NK cell fraction.

3.11 ^{51}Chromium release assay for cytotoxicity

0.5 x 10^6 of each target cell line was suspended in a minimal volume of medium and incubated with 100 micro Ci of chromium^{51} at 37°C for 60 minutes. The cells were then washed with RPMI 3 x 5 minutes at 1200 rpm in a centrifuge. NK cell effectors were incubated for 4 hours, at ratios of 20:1, 10:1, and 5: 1 with the target cell line. Chromium release was measured using a γ counter. NK cell cytotoxicity was calculated as percent lysis = [(experimental release- spontaneous release)/ (maximum release-spontaneous release)] x 100. Spontaneous release was calculated by incubating target cells without effector cells and maximal release was calculated by incubating target cells with 1% Triton X.

3.12 EBV Diagnostic Serology

EBV serological status was determined by EBV serology (IgG and IgM), to viral capsid antigen (VCA) by indirect immunoflouresence. In addition acute diagnostic samples were tested with the monospot test.

3.12.1 Monospot

A Microgen Bioproducts IM kit was used as per manufactures instructions. In brief, 25 ul of positive control or test serum was placed in each of 2 ovals of white glass slide. 1 drop of guinea pig antigen was added to one oval, and 1 drop of ox cell antigen to the other. 1 drop of horse cell suspension was added to each oval, and the slide was gently rocked. A positive result is indicated by agglutination in the oval containing guinea pig antigen, but not in the oval containing ox antigen.

3.12.2 Immunoflourescence for EBV anti VCA

All tests were carried out on serum. The serum was heat inactivated prior to testing, by heating for 20 minutes at 56°C. Test slides were prepared by placing 5 x 10^4 cells of the P3HR1 cell line in the 12 wells of a microscope slide. The test sera were diluted in PBS at 1:5 and 1:10, and were added to the slides along with control sera, from known positives and negatives. The slides were incubated for 1 hour in a humid chamber at 37°C. The slides were then washed in PBS, and incubated with 10ul of FITC conjugated rabbit anti human IgG (diluted 1:50 in PBS). The slides were incubated for a further 1hour at room temperature. The slides were washed in PBS, and mounted in PBS: glycerol. The slides were read under an epiflourescent microscope.

3.13 Quantitative EBV PCR

The EBV quantitative PCR was kindly carried out by K.McAulay following the method described by (Stevens et al., 1999) by quantitative competitive PCR (Q-PCR). The amplification reaction contained the following: 50mM KCL, 1.5mM MgCl, 10mM Tris pH 8.5, 25 pmol of each primer, one of which was biotin labelled, and 1U of taq. Cycling conditions were 4 min at 95°C; 40 cycles at 95°C, 55°C and 72°C for 1 min each; and finally 3 min at 72°C. Products were captured on a streptavidin coated plate and probed with digoxigenin labelled wild type and internal standard probes. Optical density was measured and used to calculate copy number.

3.14 Controls

3.14.1 Subjects with primary VZV infection

Four adults (2 male, 2 female, age range 28-40 years), with laboratory proven primary VZV infection, 3 of whom required hospital admission and treatment with acyclovir, were enrolled in the study. 3 cases were recruited from the Regional Infectious Disease Unit at the Western General Hospital, Edinburgh. The other case was the partner of a laboratory worker. In all cases, blood for this study was taken before commencing antiviral therapy.

3.14.2 Healthy Control Subjects

Blood was taken from 13 healthy volunteers (age range 22-30 years; 3 male, 10 female) and used for control experiments. All subjects were white Caucasian, however the age range and male:female ratios were not identical for each of the subject groups. These differences are minor and could not account for the findings in this study. Additional control data were available from UK blood donors (n=14), however Full blood counts (FBC) were not available. In addition NK cell counts were available from an additional 8 healthy controls from studies carried out by Dr Macsween.

3.15 Ethics

Ethical committee approval was obtained by the University of Edinburgh Research Ethics Committee for both the IM and VZV studies. In addition consent was obtained from the Edinburgh Students Ethics Committee for the IM study. All individuals signed a consent form, both at time of recruitment to the study, and again if they subsequently developed IM and became part of the IM cohort study (5.3.2page 101).

3.16 Statistical analysis

The Mann Whitney U test was used to test for differences in the medians of quantitative variables (e.g. CD8 counts between cases and controls). Spearman's rank correlation was used to examine associations between 2 quantitative variables. All significance tests are 2-tailed. All statistical analysis was carried out using graphpad PRISM software, www.graphpad.com 1999.

4 Results of XLP Mutation Screening

4.1 Clinical histories of cases screened for defects in the XLP gene

Clinical material was available on 10 males who had suffered a severe IM-like illness and in whom the diagnosis of XLP had been considered, on clinical grounds before identification of the gene (Table 4-1 page 89). The majority of tissue was from post-mortem examination and had been stored at -70^{O} C.

The **age range** of the cases was from 1- 34 years.

A **positive family history** was noted in 2 out of the 10 cases. In both cases this was of a brother who had predeceased the proband, and had died of a similar illness.

In 3 of the 10 cases **EBV serology** showed primary EBV infection, with positive anti VCA IgM. In addition one case may have seroconverted near the onset of the illness, as the serology recorded was carried out 16 months after the onset of the illness. Serology was not available in the other cases. In addition, EBNA positive cells were found in a lymph node in one other case, which is consistent with primary infection.

The **outcome** was known on 4 out of the 10 cases. Of these 3 cases were dead and the other one had made an uncomplicated recovery.

1 case was placed on the **XLP registry** at the time of his death, case 7.

Table 4-1 Clinical details of putative XLP cases

XLP 1 Age 8 years	16 month history of severe IM like illness with an enlarged liver, spleen and lymphadenopathy. IgM Anti VCA< 10 and IgG Anti VCA 1: 320 Anti EA 1:40. Family history and outcome unknown.
XLP 2 Age n/a	Male with history of uncontrolled EBV infection. No other clinical details available.
XLP 3 Age 4 years	3 day history of cold and sudden deterioration secondary to seizures, and subsequently died. Family history of febrile illness and rapid deterioration in deceased brother who was aged 3 at time of death.
XLP 4 Age 1 year	Acute tonsillar enlargement leading to respiratory arrest, outcome unknown. Family history of chromosomal abnormality. EBNA positive cells in lymph node.
XLP 5 Age 17 years	History of acute onset severe IM complicated by abnormal liver function, treated with acyclovir and died. No family history as adopted. Positive Monospot, Anti VCA IgM and Anti VCA IgG VCA positive and 10% of PBMC EBNA positive. Lymphoma found at post mortem.
XLP 6 Age n/a	Male infant with high EBV viral DNA load in the PBMC.
XLP 7 Age 34 years	History of acute onset severe IM complicated by hepatic, renal failure and pancytopenia. No family history of note. Treated with Fresh frozen plasma, died 13 days after admission. IgM and IgG VCA 128 and 10% of PBMC EBNA positive. At post mortem lymph node, liver, spleen, thymus, and tonsil EBNA positive. Added to XLP registry. Case history published (Crawford et al., 1979).
XLP 8 Age 14 years	History of immune dysfunction of unknown aetiology EBV viral load unremarkable. Outcome unknown.
XLP 9 Age 4 years	History of Burkitt lymphoma of bowel. Family history of pre deceased brother with lymphohistocytosis. EBV PCR unremarkable. Outcome unknown.
XLP 10 Age 24 years	Acute IM complicated by major gastrointestinal bleed requiring hemicolectomy, abnormal liver function and impaired coagulation IgM positive and IgG anti VCA 2560 & EBV genome PCR 6 x 10^3/ug DNA Full recovery made.

4.2 PCR and sequencing on XLP material

A PCR assay based direct sequencing method, derived from (Coffey et al., 1998), was used to screen for mutations in the 4 coding exons of the XLP gene (SAP/ SH2D1A) in the 10 clinical cases described in Table 4-1 page 89. The method was used as described (Section 3.6 page 72), and minimal alteration was required for exons 2, 3 and 4. Figure 4-1 shows amplification of PCR products for exon 2, 3 and 4, and subsequent cutting of gel bands prior to sequencing is shown in Figure 4-2.

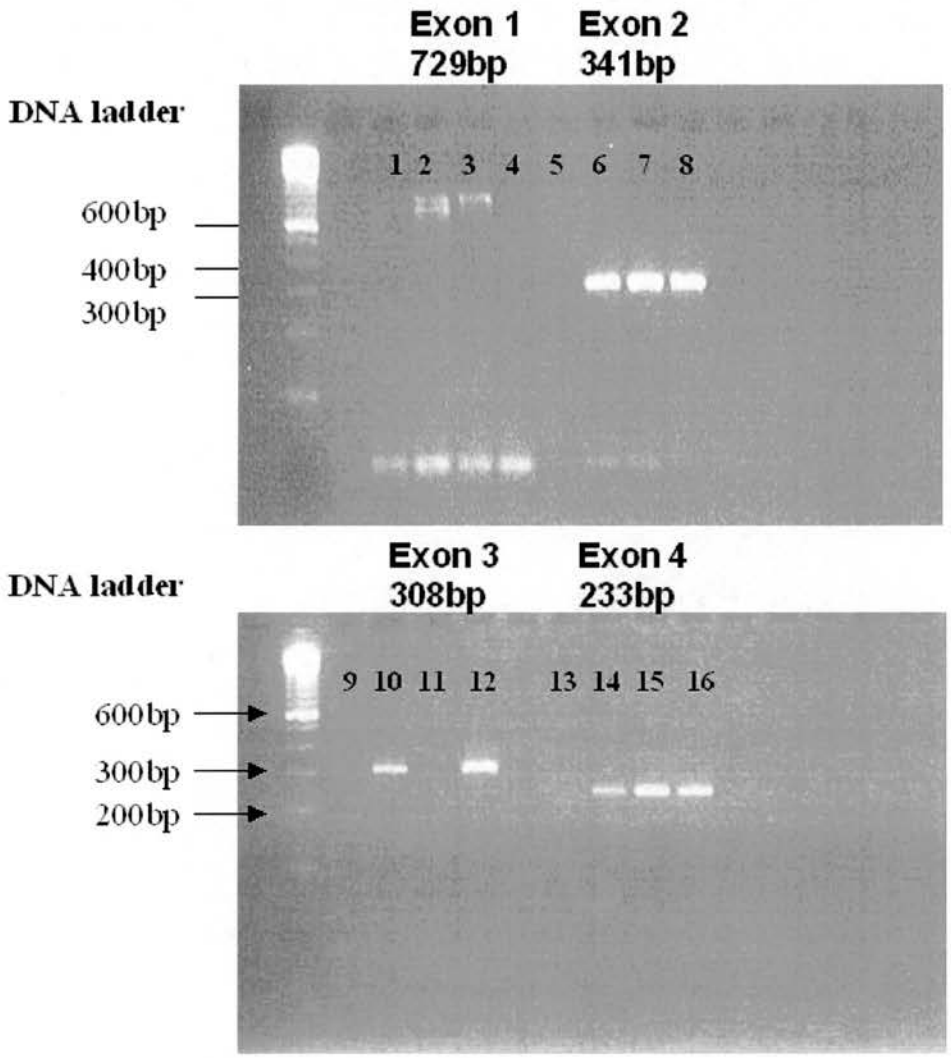


Figure 4-1 Gel picture of the 4 XLP exons.
Columns 1, 5, 9 and 13 are the negative control (water). Columns 2, 6, 10 and 14 are the positive control (genomic DNA). Columns 3, 4, 7, 8, 11, 12 15 and 16 are XLP cases. On this occasion exon 1 and exon 3 failed to amplify for an XLP case. The size of the amplified product is indicated above.

However, as exon 1 repeatedly failed to amplify a number of optimization steps were taken, described in Section 4.2.1 below. Despite these steps the exon still failed to amplify satisfactorily. Therefore a second method was used (Yin et al., 1999), as described in the Methods (Section 3.6.2 page 72), which used the same primer sequences but otherwise different reagents.

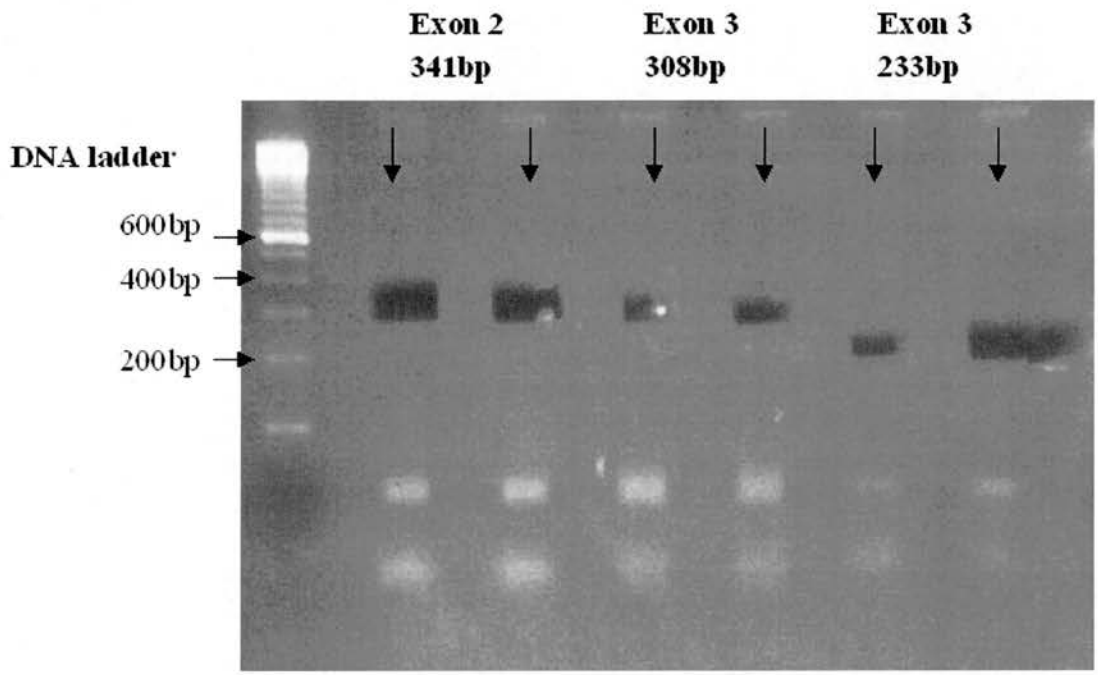


Figure 4-2 XLP exons 2, 3, and 4 after DNA bands cut for direct sequencing.

DNA was successfully amplified for exons 2, 3, and 4. The first column for each pair is genomic DNA, the second is from a case. The bands were cut prior to direct sequencing.

4.2.1 PCR optimization for exon 1

A number of optimization steps were undertaken including, alteration of annealing temperature, alteration of DNA concentration in the PCR mix, and alteration of the template concentration for the second round of the PCR (see section 4.2.1.1 page 92 onwards). Subsequently the second PCR method was used, and optimization of the gel concentration, agarose grade and voltage speed allowed identification of exon 1 bands for direct sequencing (see Section 4.2.1.4 page 93).

4.2.1.1 Annealing Temperature

The annealing temperature was verified for the exon 1 primer sets using a temperature graded PCR machine, genomic DNA as a template, and a temperatures range of 1° C between 56-65° C. Optimal bands were obtained between 60° C and 65° C (Figure 4-3 page 92). The annealing temperatures for exons 2, 3, and 4 were used as described in the published data (Coffey et al., 1998).

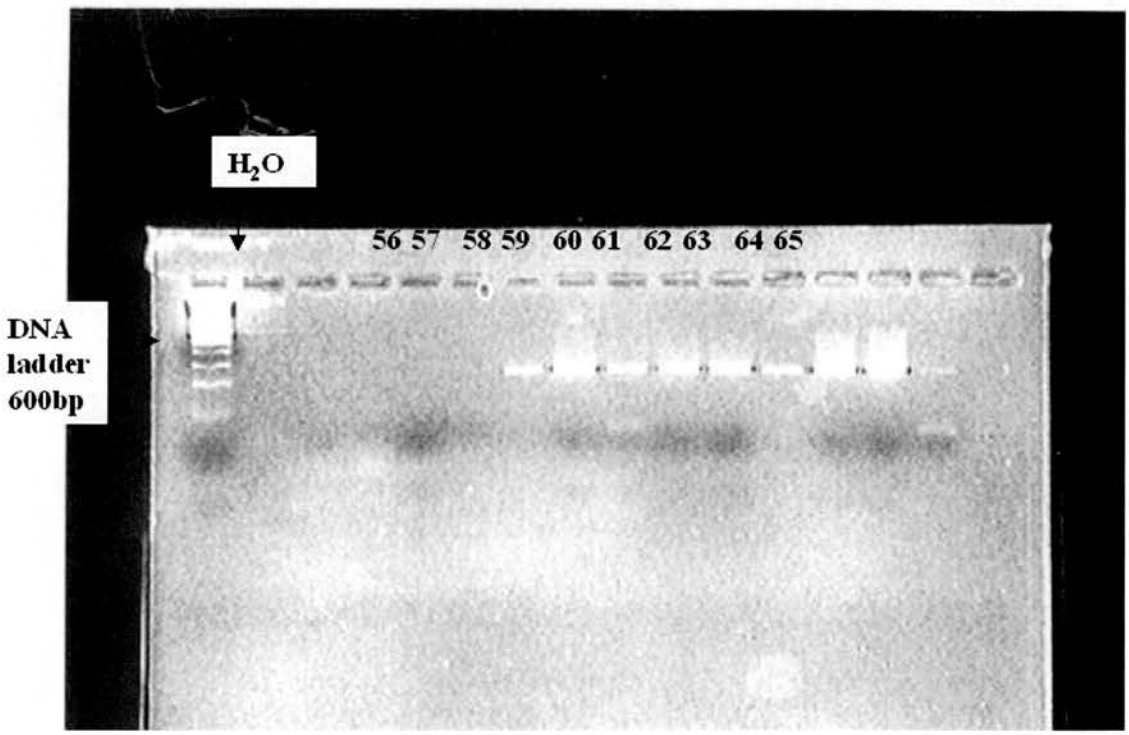


Figure 4-3 Annealing temperature for exon 1 PCR (round1).

The correct annealing temperature for exon 1 was verified using a temperature gradient of 56-65^{0}C. The temperature at which the PCR product was obtained is shown above each band.

4.2.1.2 DNA Concentration

A range of DNA concentrations in the exon 1 PCR mix were tested, from 5ng, 10ng, 20ng, 30ng, 40ng, to 50ng in 5ul of molecular grade water. Optimal bands were obtained at concentrations of 20 & 30ng/5ul of water.

4.2.1.3 Template Concentration for Second Round of PCR for exon 1

The original method described by (Coffey et al., 1998k), used a second round of PCR for exon 1, to optimize sequencing results. Using this method a second set of primers was used as described in the methods (3.6.4 page 74). A titration of the first round PCR products of, 1/2, 1/8, 1/16, 1/50, 1/100, diluted in molecular grade water was carried out. Optimal bands of 568bp, were obtained at dilutions of 1/50 and 1/100 (Figure 4-4 page 93). However, adequate sequence was not obtained. Using the second PCR method derived, the same primers as for the first round of PCR were used for the second round of PCR and satisfactory bands were obtained.

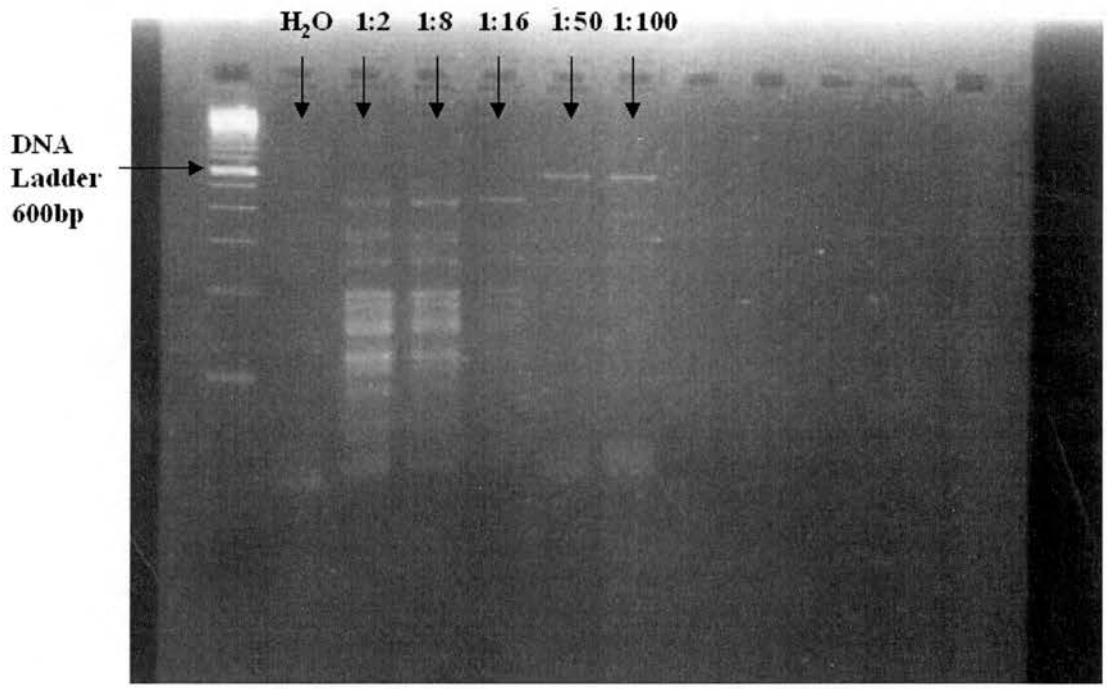


Figure 4-4 Titration of PCR template for exon 1 PCR round 2.

A gel picture is shown of a dilution series of first round PCR (568bp) products in molecular grade water, the dilution used is shown above each band.

4.2.1.4 Gel concentration, agarose grade and voltage speed.

15 ul of PCR products, and a 1kb DNA ladder were routinely run on a 2.5% agarose gel at 80 V for 60- 90 minutes, and adequate band definition for cutting was obtained for exons 2, 3 and 4. However for exon 1, 2 partially merged bands were obtained at the correct bp (729bp) marker. A higher concentration gel (5% agarose) and lower

voltage (40V) was used to increase resolution and allowed differentiation between the two bands (Figure 4-5).

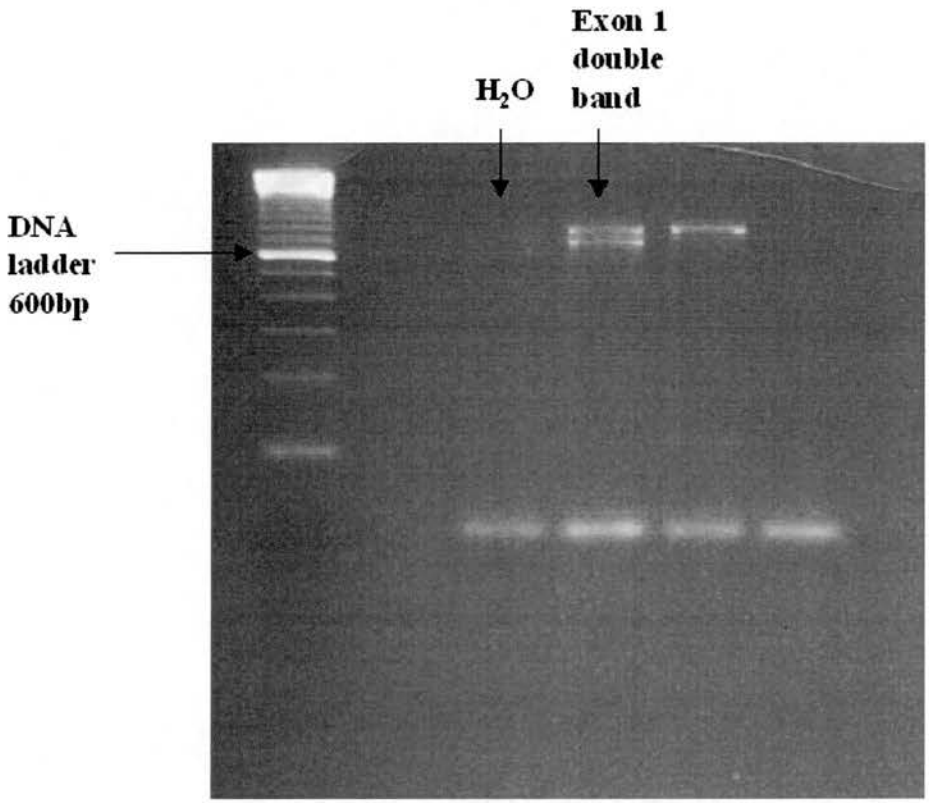


Figure 4-5 5% agarose gel showing separation of 2 bands of exon 1.

The gel picture shows the improved separation of the 2 bands obtained for exon 1 (729bp) using a high concentration gel and low voltage for running the gel.

In summary, method 2 derived from (Yin et al., 1999) allowed PCR amplification of exon 1 for the 10 cases studied. The PCR products were then used for direct sequencing as described (Section 3.6.8 page 75), with purification of gel products prior to sequencing. However, poor sequence was obtained on numerous occasions, and eventually the results were obtained in conjunction with Dr Coffey at the Sanger Centre.

4.2.2 Blast Search

Nucleotide sequences were entered into the National Center for Biotechnology (NCBI) BLAST (standard nucleotide search engine) and sequences were compared

with wild type sequence for the 4 XLP exons (Figure 4-6 page 95). Coding lengths of 436 for Exon 1, 64 bp for exon 2, 145 bp exon 3 and 128 bp exon 4 were sequenced as identified in (Coffey et al., 1998; Sayos et al., 1998). If the derived sequence contained a number of unidentified nucleotides a visual survey was made of the signal obtained using CHROMAS software, and on occasion a nucleotide could clearly be designated which the automated sequence had been unable to verify. If the sequence obtained contained nucleotides that differed from the standard BLAST sequence, or un-designated nucleotides (n) the sequencing reaction was repeated. It was decided an exon did not contain a mutation when a nucleotide sequence, which aligned 100% with the wildtype sequence, was obtained for the entire coding region.

Figure 4-6 Example of blast search on XLP 5 exon 2

```
Human DNA sequence from clone 1052M9 on chromosome Xq25. Contains
the SH2D1A gene for SH2 domain protein 1A, Duncan's disease
(lymphoproliferative syndrome) (DSHP), part of a 60S Acidic
Ribosomal protein 1 (RPLP1) LIKE gene and part of a mouse DOC4 LIK>
          Length = 134245

 Score =  589 bits (297), Expect = e-166
 Identities = 297/297 (100%)
a Strand = Plus / Plus
The 64bp coding region of exon 2 is shown in red.

Query: 23
tgtaatattaagctcaaatttaaagtatccattgttcttttggaatctttcagtaatgga 82

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 58362
tgtaatattaagctcaaatttaaagtatccattgttcttttggaatctttcagtaatgga 58421

Query: 83
agtttattctttcacaggtatcacggttacatttatacataccgagtgtcccagacagaa 142

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 58422
agtttattctttcacaggtatcacggttacatttatacataccgagtgtcccagacagaa 58481


Query: 143
acaggttcttggagtgctgaggtatagttgtatttatttttgcttctgggggtgtcaagg 202

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 58482
acaggttcttggagtgctgaggtatagttgtatttatttttgcttctgggggtgtcaagg 58541


Query: 203
aggtatttgaaatttaggctggttttataaaagagcaaattatacattattaagtattca 262

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
Sbjct: 58542
aggtatttgaaatttaggctggttttataaaagagcaaattatacattattaagtattca 58601


Query: 263
taaggtttaaatctctaaagctccaatccaaaattgttcatggatcattaagaaagc 319

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 58602
taaggtttaaatctctaaagctccaatccaaaattgttcatggatcattaagaaagc 58658
```

4.2.3 Summary of XLP Mutation Screening Results

Nucleotide sequences which aligned with the known sequence for exon 1, 2, 3, and 4 were obtained from genomic DNA. No mutations were identified in the 10 cases analyzed. Results were also available for genomic DNA from 3 healthy donors and no mutations were identified.

5 Medical Research Council seroepidemiological study of Epstein-Barr Virus in the Edinburgh Student population

5.1 Background

The ongoing seroepidemiological study of EBV in the Edinburgh student population funded by the Medical Research Council of Great Britain (MRC) has allowed access to the clinical samples and associated case histories used for the experiments and analyses detailed in chapter 6 page 109, chapter 7 page 129 and chapter 8 page 140 (see Figure 5-1 page 99). The five-year study is being undertaken in order to address the following key questions about the acquisition of primary EBV infection and the mechanisms of clinically overt seroconversion (IM) compared to silent seroconversion:

- Why do only 50% of those who seroconvert as young adults develop IM?
- Is sexual contact an important route of spread of EBV among young adults, and can virus be transmitted in sexual secretions?
- What factors cause around 10% of healthy individuals to remain EBV seronegative?
- What are the prevalence of EBV types in the community, and is each type linked to IM?

A combination of epidemiological, genetic, virological and immunological approaches have been used to answer these questions about the pathogenesis of primary EBV infection and IM. The principal investigators are Dorothy Crawford (Department of Medical Microbiology, University of Edinburgh), Anthony Swerdlow (Section of Epidemiology, Institute of Cancer Research Sutton) and Nadine Harrison, Edinburgh University Health Centre). The study is being undertaken in the student population as university students are typically of the age and social background to be at high risk of IM (see Section 2.6.1 page 28).

Moreover, as the majority of students access healthcare through a university health service, a robust mechanism for recruitment and follow up can readily be organised.

5.2 Recruitment to Seroepidemiological Study

Participants were recruited at the beginning of their first university term, in 1999 and 2000 (Figure 5-1 page 99). All new students who were due to study for 4 years or longer at Edinburgh University and who enrolled at Edinburgh University Health Centre were eligible to join the study. At recruitment participants were asked to give a blood sample and answer a detailed anonymous questionnaire assessing possible epidemiological risk factors for early or late acquisition of EBV. All participants were also advised they would be contacted 3 years after recruitment to complete the study by giving a further blood sample and completing a questionnaire. One of the aims of rebleeding the cohort after 3 years would be to identify those who had seroconverted without overt illness, and thus the rate of silent seroconversion compared to symptomatic seroconversion with IM.

All blood samples were tested for EBV status using IgG antibodies to VCA and, if this test was inconclusive, a screening test for anti EBNA IgG was used. All tests were read by 2 independent observers, and repeated if a definitive result could not be obtained. All seronegatives were also negative by PCR testing.

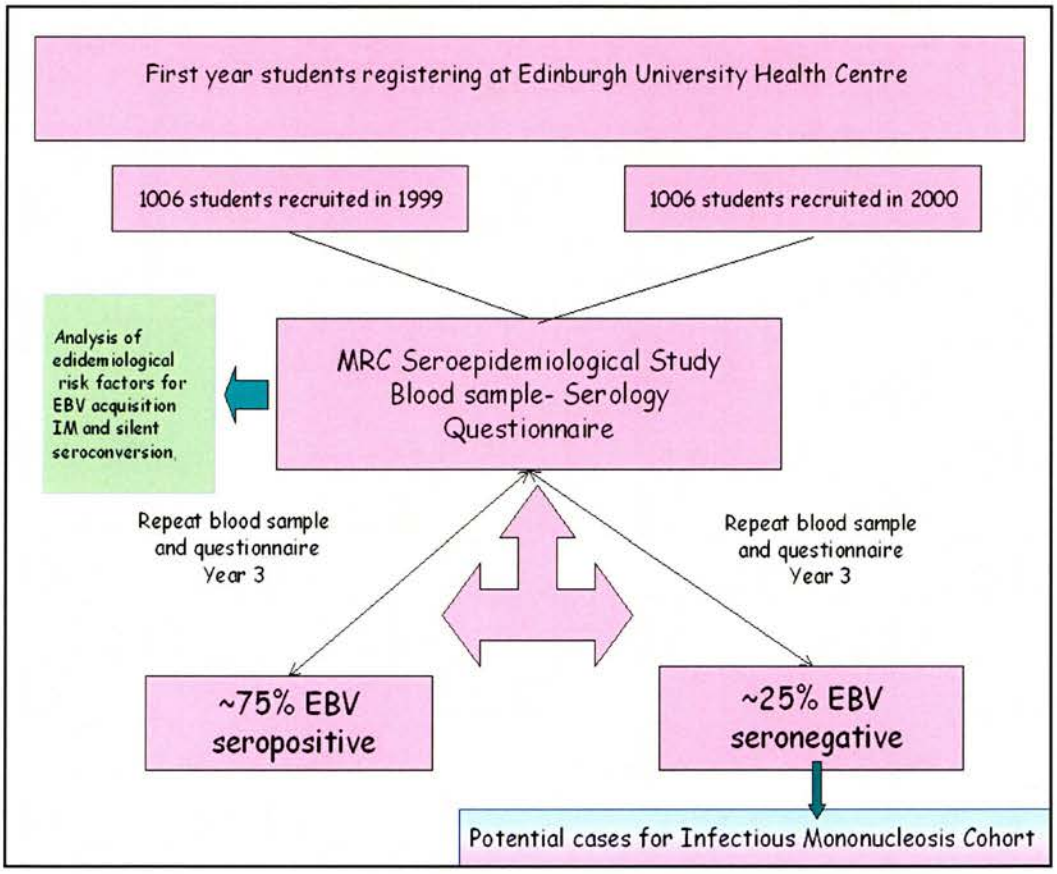
All participants were subsequently informed of their serological status, and thus if they remained at risk of developing IM. All seronegative participants were advised of the symptoms of IM, including prolonged sore throat, swollen glands, fever, and fatigue, and asked to contact Edinburgh University Health Centre if they were concerned that they had developed IM. Our laboratory carries out all routine diagnostic testing of EBV serology for Edinburgh University Health Centre, and thus all seronegative participants developing IM were readily identified.

This prospective study on IM has enabled the establishment of a large IM cohort with detailed data on the clinical sequelae of IM, both at diagnosis and over the course of the illness. Alongside the clinical assessment, there has been concurrent collection of clinical samples including viable PBMC, DNA, serum and throat washings. At present the study has focused on 2 aspects of the pathogenesis and

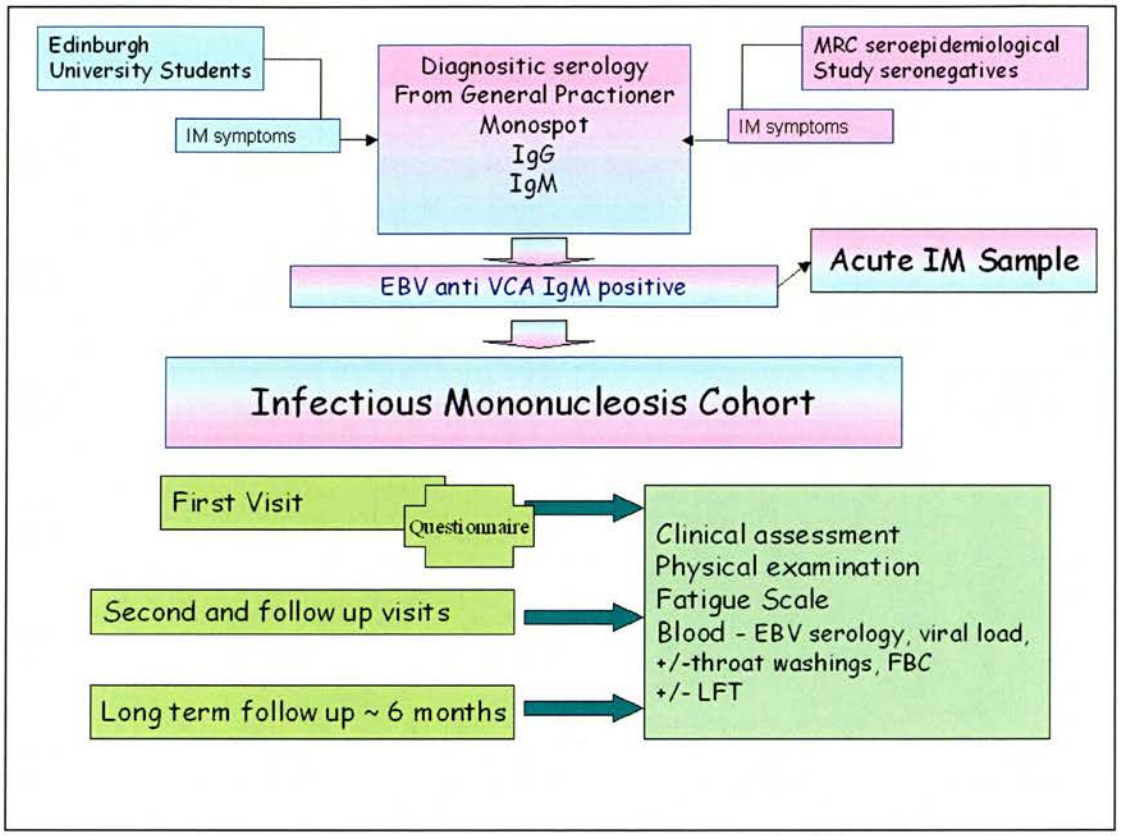
clinical outcome of IM: firstly a detailed case control study reassessing the clinical course of IM, in particular in relation to fatigue, and impact on outcome of university studies (Dr Macsween); secondly, an assessment of immune activation in IM, both at diagnosis and during convalescence, and in particular how immune activation correlates with clinical illness. Figure 5-1 below gives a visual overview of the MRC study.

Figure 5-1 (a, b and c) Overview of MRC study and IM cohort

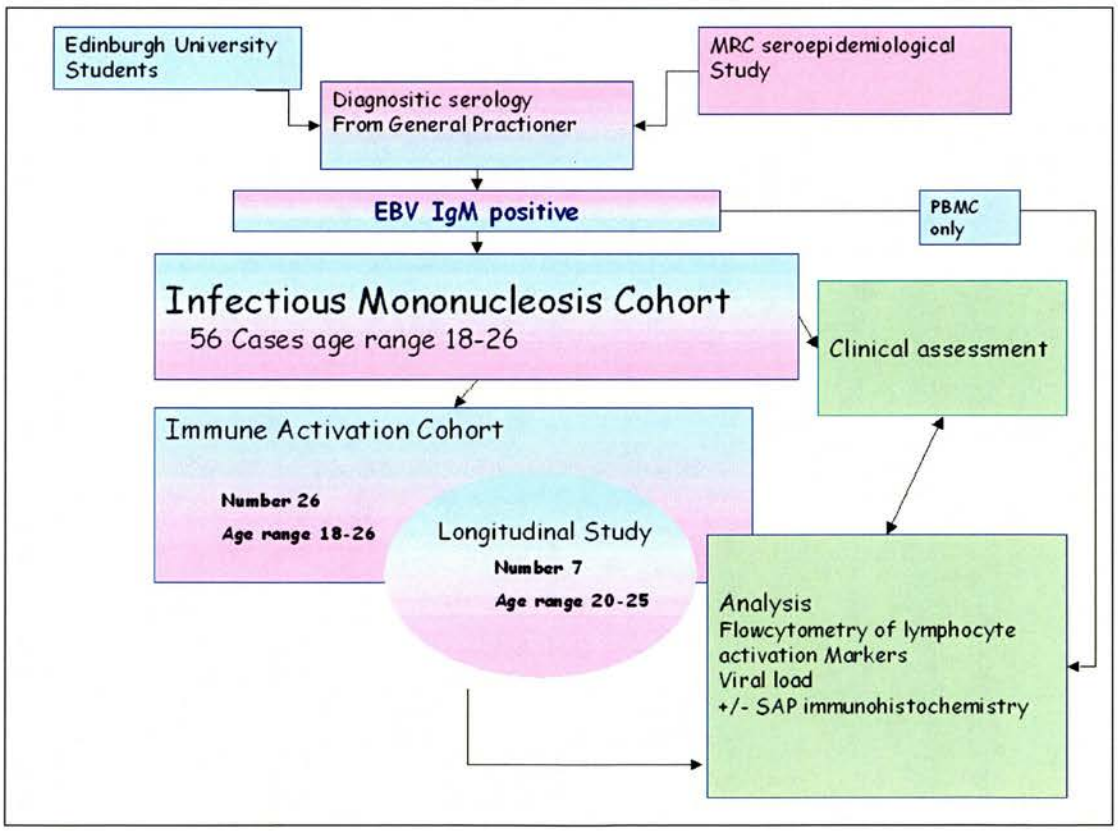
a



b


Edinburgh University Students
IM symptoms
Diagnositic serology From General Practioner
Monospot
IgG
IgM
MRC seroepidemiological Study seronegatives
IM symptoms
EBV anti VCA IgM positive
Acute IM Sample
Infectious Mononucleosis Cohort
First Visit
Questionnaire
Second and follow up visits
Long term follow up ~ 6 months
Clinical assessment
Physical examination
Fatigue Scale
Blood - EBV serology, viral load,
+/-throat washings, FBC
+/- LFT

c


Edinburgh University Students
Diagnositic serology From General Practioner
MRC seroepidemiological Study
EBV IgM positive
PBMC only
Infectious Mononucleosis Cohort
56 Cases age range 18-26
Clinical assessment
Immune Activation Cohort
Number 26
Age range 18-26
Longitudinal Study
Number 7
Age range 20-25
Analysis
Flowcytometry of lymphocyte activation Markers
Viral load
+/- SAP immunohistochemistry

5.3 Study Design for IM Cohort

5.3.1 Criteria for Inclusion in the IM Cohort

All students (including both those previously registered with the MRC study and those who have not) who attend the Edinburgh University Health Centre with symptoms suggestive of IM undergo EBV serological testing (IgM and IgG antibodies to VCA and monospot). In addition, at the GP's discretion, a full blood count (FBC) and Liver Function Tests (LFT) are sent for routine diagnostic testing. All those with positive IgM antibodies to VCA were suitable for inclusion in the IM Cohort and were contacted by a study doctor. The diagnostic criteria for IM was a positive EBV VCA IgM antibody test, +/- a positive monospot; those who were only monospot positive were excluded. In addition, a number of the participants in the MRC Seroepidemiological study developed IM, which was diagnosed outwith the University Health Service. These people were asked to contribute to the study on return to Edinburgh, and a retrospective assessment of the illness was made. However, only cases in whom concurrent clinical samples and data were available at the time of acute illness were included in the immune activation cohort which is defined in the following section.

5.3.2 Definition of the IM cohort, immune activation cohort & longitudinal cohort

In order to clarify the relationship between the cases recruited with acute IM, and the subset in whom more detailed investigation were carried out, including immune activation studies, each cohort has been defined. The demographic and clinical features of the IM cohort and Immune activation cohort are recorded in Table 5-1 (page 102, and see Figure 5-1 page 99).

5.3.2.1 IM Cohort

The IM cohort consists of all participants recruited with acute IM from September 1999 to December 2002. 56 cases were included in this study.

5.3.2.2 Immune Activation Cohort

The immune activation cohort is a subgroup of 26 cases from the IM cohort in whom viable cells were available at diagnosis with concurrent record of clinical features, and in whom immune activation studies could be carried out.

5.3.2.3 Longitudinal Cohort

The longitudinal cohort is a subgroup of 7 cases from the immune activation cohort in whom, in addition to the analysis made at diagnosis, samples were available for immune activation studies with concurrent clinical details during recovery and at around 6 months after diagnosis.

Table 5-1 Demographic features of cohorts

Number of cases	IM cohort	56
	Immune activation cohort	26
Age	IM cohort	Range 18-26 years
	Immune activation cohort	Range 18-26 years
Sex	IM Cohort	Male 29 Female 27
	Immune activation cohort	Male 12 Female 14

5.4 Clinical assessment of the IM Cohort.

After recruitment to the IM Cohort Study all participants were followed up by the study doctor, and the clinical assessment was carried out as detailed in Table 5-2 (page 103). A clinical proforma was designed in order to record the same clinical information, in an objective manner, in each case and monitor the course of the illness. In order to standardise data collection, the date on which blood was drawn for EBV serology was recorded as the date of diagnosis, and subsequent study visits were recorded as visit 1, visit 2 etc. The first study visit was made as soon as possible after diagnosis. When possible, cases were followed up at 2 weeks and 1 and 2 months, or until they had returned to their pre illness activity level, normally defined by return to full time studies. All patients were recalled at around 6 months.

However, not all time points were met, often due to individuals returning to full-time study or to their parental home.

Table 5-2 Schedule of Follow up and investigations in the IM Cohort

<table>
<tr><td>Diagnostic blood (20 ml EDTA)</td><td colspan="2">Monospot and IgM and IgG to VCA

Storage of viable PBMC, serum and DNA</td></tr>
<tr><td rowspan="3">First follow up visit</td><td colspan="2">Information sheet and consent

Questionnaire (confidential) – family, social, work, travel and sexual risk factors for EBV acquisition

Clinical proforma- details of worst point in illness and current point

Examination proforma and Fatigue scale – fatigue at worst point in illness, current point and when well.</td></tr>
<tr><td>Blood</td><td>FBC & LFT

EBV serology & viral load

Storage of viable PBMC, serum and DNA</td></tr>
<tr><td>Throat washings</td><td>EBV strain analysis</td></tr>
<tr><td>Second and subsequent follow up visits</td><td colspan="2">Clinical proforma- details of worst point in illness and current point

Examination proforma

Fatigue Scale

Blood as visit one</td></tr>
</table>

5.5 Results of clinical assessment of IM

In order to examine the relationship between illness severity and immune activation we needed to develop a robust and objective measurement of clinical features. The clinical proforma in the appendix shows which features of the illness were recorded

in each case. From these data we identified clinical parameters that were both objective and reflected the variation in illness severity experienced in the cohort. Therefore only the parts of the clinical assessment which were used in the immune activation study (Chapter 6 page 109 and Chapter 8 page 140) are discussed below.

Results are shown for both the IM cohort (56 cases) and the immune activation cohort (26 cases). The number of visits per case ranged from 1 –7.

5.5.1 Time to first follow up and definition of worst symptoms

The typical course of IM consists of a short prodrome, followed by an acute phase, followed by a variable period of recovery. We found that participants tended to visit the GP around the time of the acute phase, and this is when the diagnostic blood was taken. However, at the time an individual was seen by the study doctor at visit one, the most acute symptoms may have resolved, and therefore both the most severe symptoms experienced and the current symptoms were recorded.

The first follow up was carried out as soon as possible after diagnosis, and for the immune activation cohort the range was 2-16 days, with a mean of 9 days. With three exceptions, the worst point of the illness was within 6 days of diagnosis.

5.5.2 Definition and analysis of sore throat

Sore throat is one of the hallmarks of IM, and severe tonsillar swelling and obstructive symptoms is one of the most common causes of hospital admission. Mild sore throat was defined as being able to swallow a normal diet at the worst point of illness, and severe as unable to swallow normal diet or more extreme symptoms. The results are shown in Table 5-3 (page 104), the data were available in all cases.

Table 5-3 Analysis of sore throat in acute IM

Symptoms of Sore throat		Number of cases	% of cases
IM Cohort	Severe	21	38
	Mild	35	64
Immune Activation Cohort	Severe	12	46
	Mild	14	54

5.5.3 Definition and analysis of fever, lymphoadenopathy and rash

Participants were able to clearly and consistently identify whether they had experienced a number of physical symptoms including fever (measured without using a thermometer), swollen glands, rash, anorexia and headache. The results are shown in Table 5-4 (page 105) the number of cases who experienced the symptom is shown over the total number in which data were recorded.

Table 5-4 Analysis of clinical features of IM

Clinical Feature	Cohort	Number of cases	% of cases
Cases with fever	IM Cohort	42/56	75
	Immune Activation Cohort	21/26	81
Cases with Headache	IM Cohort	37/56	66
	Immune Activation Cohort	15/26	57
Cases with Anorexia	IM Cohort	44/56	78
	Immune Activation Cohort	22/26	85
Cases with Swollen glands	IM Cohort	53/56	95
	Immune Activation Cohort	26/26	100
Cases with Rash	IM Cohort	5/51	10%
	Immune Activation Cohort	5/26	19%

5.5.4 Assessment of fatigue

Fatigue is a common symptom of IM, and prolonged fatigue is a frequent complication. Moreover, there has been considerable interest in the relationship between IM and chronic fatigue syndromes, and in our cohort the impact residual fatigue has on an individual's ability to complete their university studies.

Ideally, the assessment of fatigue should be objective and allow comparison over time in an individual, and also comparison between cases with very different pre illness levels of activity and work patterns. A detailed review of the literature did not identify any fatigue scales that fulfilled these criteria and were suitable for use in this

cohort (Monga et al., 1999; Behrenz and Monga, 1999). We therefore devised a fatigue scale that assessed 3 parameters of fatigue (see appendix).

- Physical function, such as ability to dress or leave home
- Impact on university attendance and studies
- Individuals subjective feeling of fatigue
- All parameters were compared to an individual's function when well. As there is a strong psychological element to the impact an illness has on an individual's ability to function, it would be difficult to investigate correlations between these parameters and immune function. We therefore focused on the relationship between physical function at worst point in illness, e.g. ability to leave home, and correlated this with parameters of immune response. The results are shown in Table 5-5 (page 106).

Table 5-5 Analysis of Fatigue in IM

		Number of cases	% of cases
Cases able to leave home at worst point of illness	IM Cohort	35/55	64
	Immune Activation Cohort	17/26	65
Cases able to attend university full time	IM Cohort	6/57	10
	Immune Activation Cohort	6/26	23

5.5.5 Definition of recovery and long term follow up

Recovery from illness was defined as when an individual could return to their pre - morbid level of function, and this usually meant return to full time studies. We recalled all participants at around 6 months to obtain a blood sample and to monitor recurrence of symptoms. Of the 26 cases in the immune activation cohort, the prodrome was a median of 6 days with a range of 0-31 days. The severe period was a

median of 7 days with a range of 2-14, and the overall length of illness was a median of 32 days with a range of 5 days to over 6 months.

A summary of the clinical features of the 26 cases in the immune activation cohort is provided in Table 5-6.

Table 5-6 Summary of clinical details for 26 cases of IM in the immune activation cohort

	Age	Sex	Attendance at university	Severity of sore throat at diagnosis	Fever	Able to leave home*	Rash	Viral load**
1	22	F	Part time	Severe	Yes	No	No	4554
2	20	F	Part time	Mild	Yes	Yes	Yes	5841
3	19	F	Full time	Mild	No	No	No	82
4	22	M	Part time	Severe	Yes	No	No	3498
5	22	F	Part time	Severe	Yes	Yes	No	14982
6	20	M	Full time	Mild	No	Yes	No	2376
7	20	M	Full time	Severe	Yes	Yes	No	NK
8	25	F	Part time	Mild	Yes	Yes	Yes	867
9	20	M	Part time	Mild	No	Yes	No	3036
10	20	M	Full time	Severe	Yes	No	No	Na
11	24	M	Part time	Mild	Yes	Yes	Yes	14982
12	26	M	No attendance	Mild	Yes	Yes	Yes	2673
13	21	F	No attendance	Severe	Yes	Yes	No	2409
14	21	F	Part time	Severe	Yes	No	No	11913
15	20	M	Part time	Severe	Yes	Yes	No	Na
16	20	F	Part time	Mild	Yes	No	No	2771
17	18	F	Part time	Mild	Yes	No	No	12886
18	218	M	Full time	Mild	Yes	Yes	No	8007
19	19	M	Full time	Severe	Yes	Yes	No	16422

20	20	M	On holiday	Severe	Yes	Yes	No	6868
21	19	F	Part time	Severe	Yes	No	No	4420
22	19	F	Part time	Mild	No	Yes	No	7514
23	22	F	On holiday	Mild	No	Yes	No	646
24	19	F	Part time	Mild	Yes	Yes	Yes	0
25	22	M	On holiday	Mild	Yes	Yes	No	2550
26	21	F	Part time	Severe	Yes	No	No	49249

Na = data unavailable F= female. M= Male

* = able to leave home at worst point in illness

**=EBV genome copies per 10^6 PBMC

6 Immune activation and clinical events in Infectious Mononucleosis

It is well established that there is a dramatic lymphocytosis in acute IM, principally consisting of CD8 positive T cells. However, it is not clear what changes occur in other lymphocyte subsets, how long the lymphocyte numbers remain raised, and how this relates to the severity of illness. We analysed lymphocyte subsets by flow cytometry in 26 cases at the time of diagnosis of acute IM (Table 5-6 page 107). Data were compared with results from healthy controls, but as total lymphocyte counts were not available on all controls, only the first 14 cases were used for analysis when lymphocyte counts were required for comparison purposes. Ranges and medians are shown in the text; further data are included in the appendix.

6.1 Analysis of lymphocyte subsets in acute IM

6.1.1 Total lymphocyte counts, CD3, and CD8 cell counts were all significantly elevated at diagnosis of IM

The total lymphocyte count, CD3 and CD8 counts were significantly elevated at diagnosis of IM ($p< 0.01$ for all subsets, for ranges see Table 6-1 page 110). The median value for total lymphocyte counts was 7.19 x 10^6 per ml for cases and 1.67 x 10^6 per ml for controls; for CD3 counts the median value was 5.07 x 10^6 per ml for cases and 1.00 x 10^6 per ml for controls; and for CD8 counts the median was 4.40 x 10^6 per ml for cases and for controls 0.48 x 10^6 per ml. Neither CD4 nor B cell counts were significantly altered.

The spectrum of lymphocytosis was wide, which may reflect the variable clinical course of IM.

Table 6-1 Lymphocyte subsets in acute IM

	Cell Counts x 10^6 per ml		
Cell type	At diagnosis of acute IM	At first review of IM	Control Subjects
Total Lymphocyte	1.98-12.65 **	1.45-2.33	1.23-2.41
CD3+ Lymphocyte	0.89-9.62 **	0.96-1.62	0.49-1.79
CD4+ Lymphocyte	0.31-0.78	0.35-0.52	0.11-0.90
CD8+ Lymphocyte	0.58-9.43 **	0.46-0.62	0.31-0.89
B Cell	0.30-0.90	0.13-0.54	0.17-1.54

** median values were significantly different to control values ($p<0.01$)

6.1.2 Acute IM causes significant activation of the SAP/SLAM/CD244 activation pathway

The clinical features of IM have been attributed to the CD8 lymphocytosis. However, the activation status of the cells has not been fully investigated. The expression of the lymphocyte activation molecules CD244 and SLAM was analysed by flow cytometry in the same 26 cases (Table 5-6 page 107).

6.1.2.1 *At diagnosis of acute IM numbers of T cell subsets expressing CD244 were significantly elevated*

At diagnosis of acute IM numbers of CD3, CD4 and CD8 T cell subsets expressing CD244 were all significantly elevated; ($p< 0.01$ for all subsets and for ranges see Table 6-2 page 111). The median number of CD3 positive cells expressing CD244 was raised at 3.95 x 10^6 per ml for cases compared to 0.27 x 10^6 per ml for controls. The median values for CD4/CD244 double positives, was 0.2 x 10^6 per ml for cases and 0.04 x 10^6 per ml for controls, and the median value for CD8/CD244 double positives was also markedly raised at 3.17 x 10^6 per ml for cases compared with 0.22 x 10^6 per ml for controls (Figure 6-1 page 113). The median percentages of CD3 (median 77%), CD4 (median 30%) and CD8 (median 82%) cells expressing CD244 in IM cases were also increased over control median values of 14%, 9% and 51% for

CD3, CD4, and CD8 respectively ($p<0.001$) for all percentage data sets (Figure 6-2 page 114, Figure 6-3 page 115 and Figure 6-4 page 116). As previously reported, CD244 was not expressed on B cells, and was constituently expressed on NK cells (Nakajima et al., 1999).

Table 6-2 Expression of CD244 in acute IM

	At diagnosis of acute IM		Control Subjects		First follow up of IM
	Cell number expressing CD244 x 10^6 per ml	% of subset expressing CD244	Cell number expressing CD244 x 10^6 per ml	% of subset expressing CD244	% of subset expressing CD244
CD3	0.40-9.33**	35-97***	0.09-0.81	0-33	21-47**
CD4	0.05-0.48**	8-80***	0.02-0.07	1.75-17	7-75*
CD8	0.45-8.77***	25-97***	0.14-0.67	33-77	50-71*

*** median values were significantly different to control values ($p<0.001$)

** median values were significantly different to control values ($p<0.01$)

* median values were significantly different to control values ($p<0.05$)

6.1.2.2 At diagnosis of IM, numbers of CD3, and CD8 T cells expressing SLAM were all significantly elevated

At diagnosis of IM, median numbers of CD3, and CD8 T cells expressing SLAM were all significantly elevated ($p< 0.01$ and for ranges Table 6-3 page 112 and see Figure 6-1 page 113).

The median number of CD3 cells expressing SLAM was 2.17 x 10^6 per ml for cases, compared to 0.06 x 10^6 per ml for controls. For CD8/SLAM double positive cells, the median value for cases was 1.86 x 10^6 per ml compared to 0.06 x 10^6 per ml for controls. The percentage of IM CD8 cells (median 60%) expressing SLAM was also significantly different from controls (median 41%) ($p<0.001$) (Figure 6-4 page 116).

The number of CD4 cells expressing SLAM in IM cases was not significantly elevated (median value of 0.21 x 10^6 per ml compared to a control value of 0.09 x10^6 per ml), but the percentage of CD4 cells expressing SLAM, and thus an activated phenotype, was significantly increased (median 60% compared to a control median

of 31%) ($p<0.05$) (Figure 6-3 page 115). As CD4 cells are central to the TH1/TH2 balance of the immune response, the change in activation status (despite minimal changes in total cell numbers) may be an important influence on the balance of cytokine release in EBV infection.

Table 6-3 Expression of SLAM in acute IM

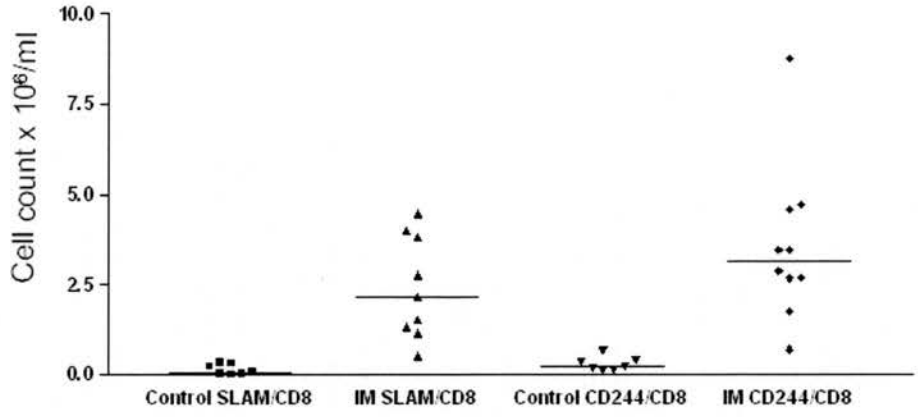
	At diagnosis of acute IM		Control Subjects		First follow up of IM
	Cell number expressing SLAM x 10^6 per ml	% of subset expressing SLAM	Cell number expressing SLAM x 10^6 per ml	% of subset expressing SLAM	% of subset expressing SLAM
CD3	0.77-5.6***	11-100	0.0-0.68	0-92	27-85
CD4	0.07-0.48	17-100*	0.0-0.38	3-92	31-91
CD8	0.3-4.53***	26-92**	0.0-0.35	0-92	35-70*

*** median values were significantly different to control values ($p<0.001$)

** median values were significantly different to control values ($p<0.01$)

* median values were significantly different to control values ($p<0.05$)

I



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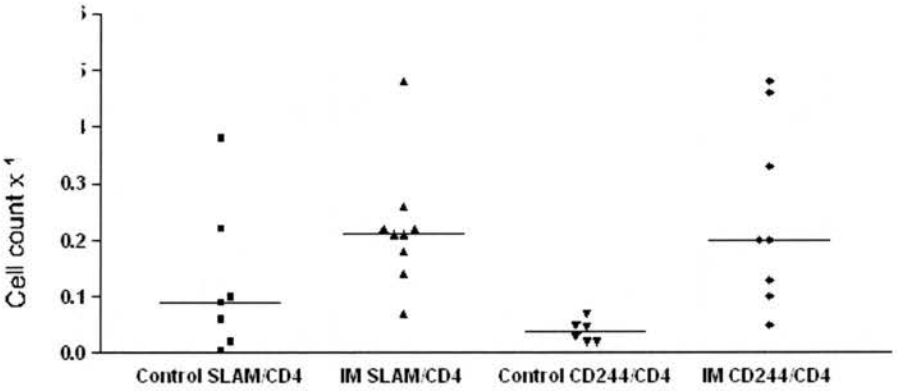


Figure 6-1 Upregulation of SLAM/CD244 on peripheral blood lymphocytes during acute IM.

I The number of peripheral blood CD8 cells expressing either SLAM (median 1.86 $x10^6$ per ml) or CD244 (median 3.17 x 10^6 per ml) in acute IM was significantly higher ($p<0.001$) compared to control values. Bars show median values.

II The number of peripheral blood CD4 cells expressing CD244 (median 0.2 x 10^6 per ml) was significantly higher in acute cases of IM, ($p<0.01$) compared to controls. The median number of CD4 cells expressing SLAM (median 0.21 x 10^6 per ml) was not significantly different from the control values. Bars show median values.

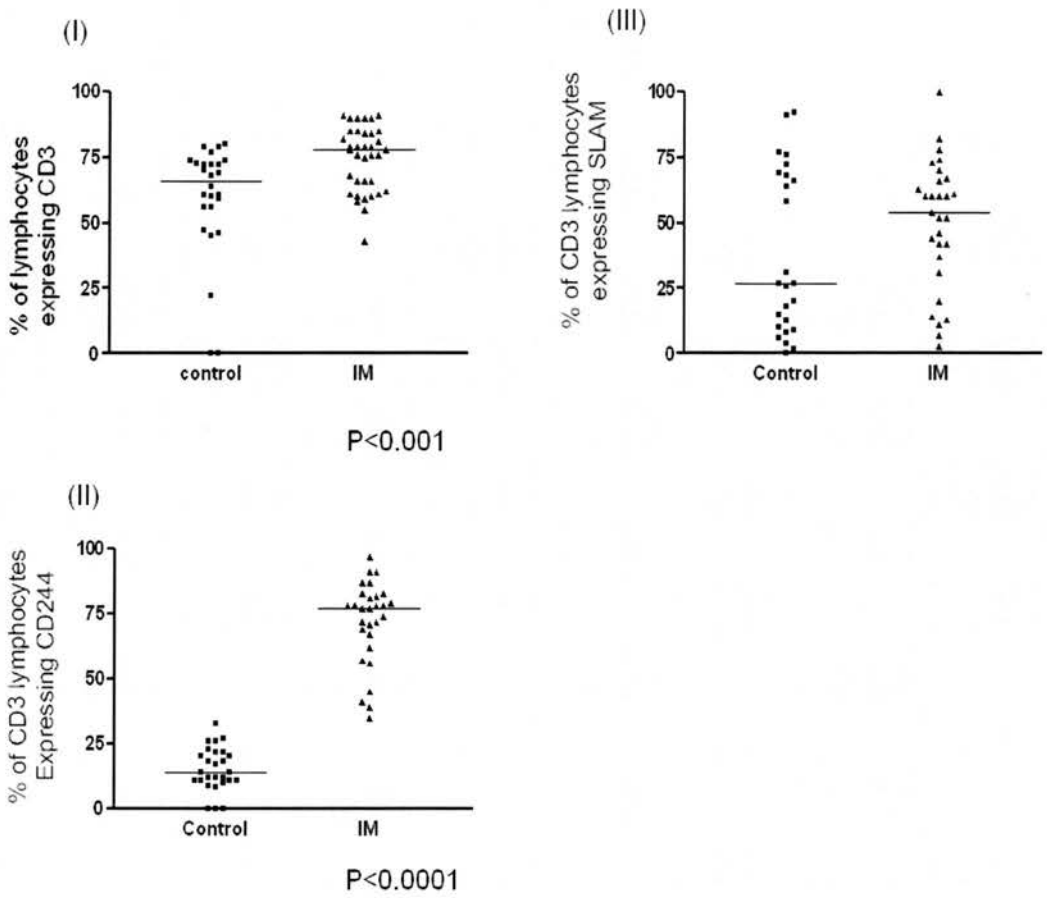


Figure 6-2 (I, II and III) Percentages of CD3 lymphocyte subsets are higher in acute IM compared to healthy controls

In each plot, bars show the median values, and the difference between the 2 medians was statistically significant in plots where the p value is shown. All values refer to peripheral blood. The same format is followed in Figure 6-3 (page 115) and Figure 6-4 (page 116).

I Shows the percentage of lymphocytes expressing CD3. The median for healthy controls was 66% and for cases was 76%.

II Shows the percentage of CD3 lymphocytes which express CD244. The median for healthy controls was 14% and for cases was 77%.

III Shows the percentage of CD3 lymphocytes which express SLAM. The median for healthy controls was 27% and for cases was 54%.

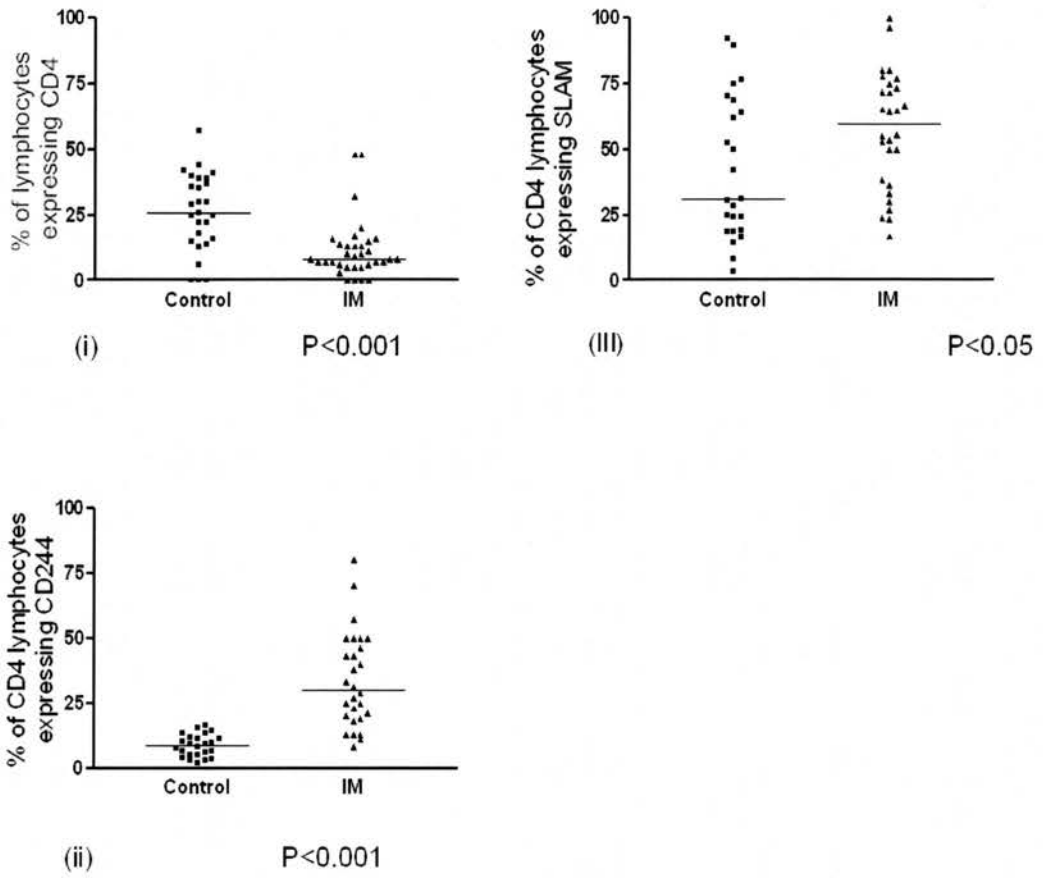


Figure 6-3 (I, II and III) Percentage of CD4 lymphocyte subsets expressing CD244 or SLAM is higher in acute IM than in healthy controls

I shows percentage of CD4 lymphocyte subsets in both healthy controls and cases of acute IM. The median for healthy controls was 39% and for cases was 15%.

II shows the percentage of CD4 lymphocytes which express CD244. The median for healthy controls was 9% and for cases was 30%.

III shows the percentage of CD4 lymphocytes which express SLAM. The median for healthy controls was 31% and for cases was 60%.

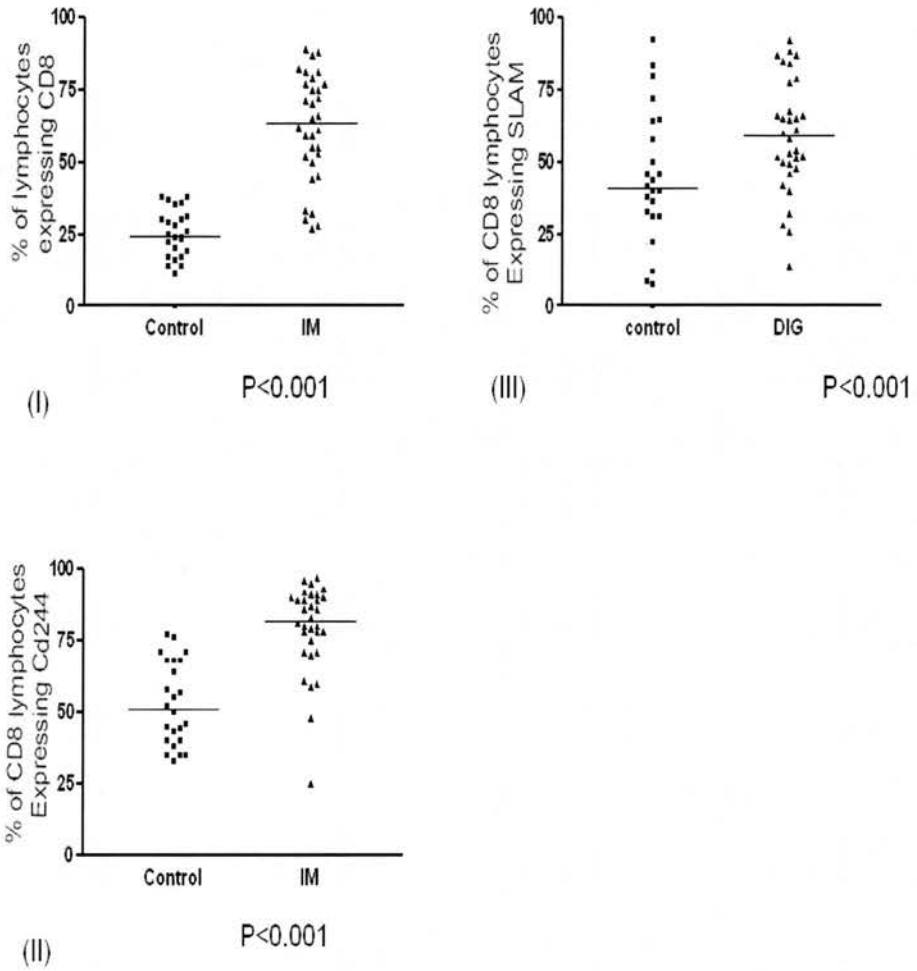


Figure 6-4 (I, II and III). Percentages of CD8 lymphocyte subsets expressing SLAM or CD244 are higher in acute IM compared to healthy controls

I Shows the percentage of lymphocytes expressing CD8. The median for healthy controls was 24% and for cases was 64%.

II Shows the percentage of CD8 lymphocytes, which express CD244. The median for healthy controls was 51% and for cases was 82%

III Shows the percentage of CD8 lymphocytes, which express SLAM. The median for healthy controls was 41% and for cases was 60%.

6.2 Longitudinal analysis of IM immune response

We carried out a longitudinal study in a group of 7 patients, (cases 1, 4, 5, 9, 10, 11 and 12 from Table 5-6 page 107) during the course of IM in order to relate the clinical symptoms to changes in lymphocyte subsets in the peripheral blood. Cases were followed up until they had returned to their pre-morbid activity level.

6.2.1 Lymphocytosis in acute IM falls rapidly despite ongoing clinical symptoms

At diagnosis 86% of IM patients had raised lymphocyte counts. In contrast, at the first review 9-16 days later, and despite features of ongoing clinical illness, all of the total lymphocyte counts and all the CD3 and CD8 subsets had returned to within the range established for controls and remained within this range for the duration of the illness in all subjects (Table 6-1 page 110). Thus the prolonged clinical features of IM were not simply a reflection of a sustained elevation in lymphocyte numbers in the peripheral blood.

6.2.2 CD8 cells maintain an activated phenotype over the duration of clinical illness

In order to investigate why clinical features such as lymphadenopathy remained when absolute lymphocyte counts rapidly return to normal following diagnosis, the level of activation molecules expressed on T cell subsets was examined. At the time of first follow up total numbers of CD3 and CD8 cells expressing CD244 had fallen in each case. However, the median percentages of CD3, CD4 and CD8 cell subsets expressing CD244 remained significantly elevated above that of controls ($p < 0.05$) The median percentage of CD3 cells expressing CD244 was 40% for cases, and 14% for controls, the median percentage of CD8 cells expressing CD244, was 64% for cases and 51% for controls, and for CD4/CD244 double positives was 14% for cases and 9% for controls (Table 6-2 page 111, Figure 6-5 page 119 and Figure 6-6 page 120 for data on case 9).

Both the number of CD8 cells expressing SLAM (median 0.32 x 10^6 per ml) and the percentage (median 59%) of cells expressing SLAM at first follow up, were significantly elevated above controls, ($p< 0.05$, Table 6-3 page 112). Neither the numbers of CD4 cells expressing SLAM (median 0.23 x 10^6 per ml) nor the percentage of SLAM/CD4 double positive cells (median 46 %) were significantly different from controls at this time point.

At follow up closest to 1 month, (range 20-42 days) 5 out of 5 cases studied remained unwell with ongoing fatigue in all subjects. In addition 2 cases had physical signs; one had enlarged tonsils and the other enlarged glands. The

percentage of SLAM/CD8 double positives and CD244/ CD8 double positive cells remained elevated in 4 out of the 5 cases. This prolonged expression of CD244 and SLAM on CD8 cells indicates that T cells remain activated over the course of the clinical illness, which may explain the extended clinical symptoms.

At the final follow up (range 59-80 days following diagnosis of IM), 6 cases had made a full recovery, but 1 case still suffered fatigue (Case 4). In all cases lymphocyte counts and CD3, CD8 and CD4 subsets were within the range for controls. Interestingly, expression of both CD244 and SLAM on CD8 cells had fallen in all cases, except in the case with sustained fatigue in whom levels of CD244 and SLAM remained high with 60% of CD8 cells expressing CD244 and 71% of CD8 cells expressing SLAM.

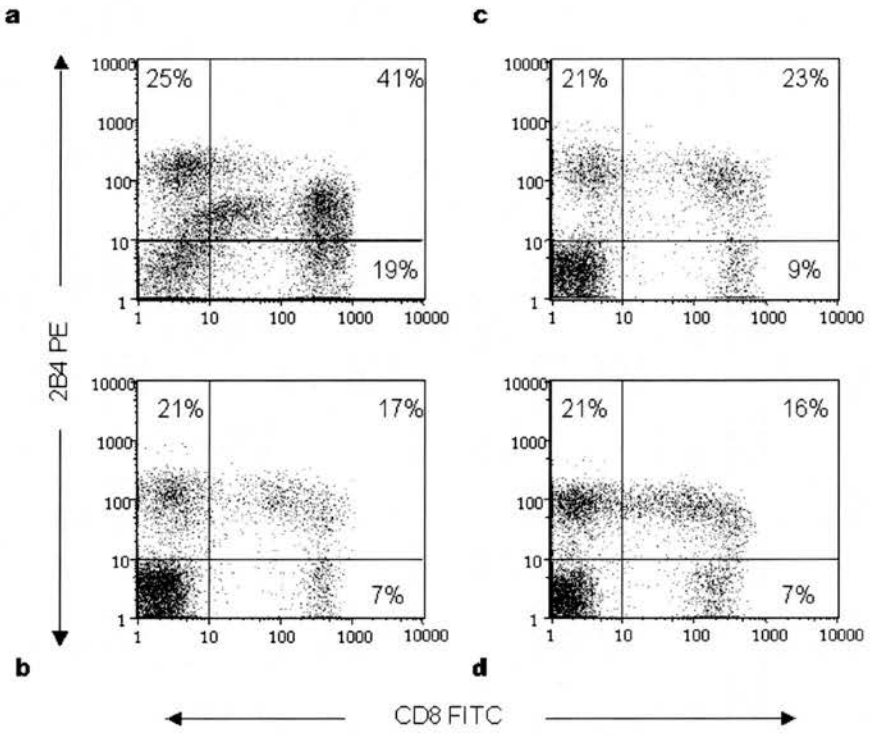


Figure 6-5 (a-d) **Flow cytometric analysis of lymphocytes showing increased expression of CD244/CD8 on lymphocytes.** In the above plots the cells were gated on lymphocytes. However, when the analysis was carried a further CD3 positive gate was used in addition.

The data shown are from case 9 from Table 5-6 page 107.

(a) At diagnosis VL= 3036

(b) 16 days after diagnosis VL=78

(c) 42 days after diagnosis VL= 1

(d) 6 months after diagnosis VL=241 (VL = viral load in EBV genomes /10^6 PBMC.)

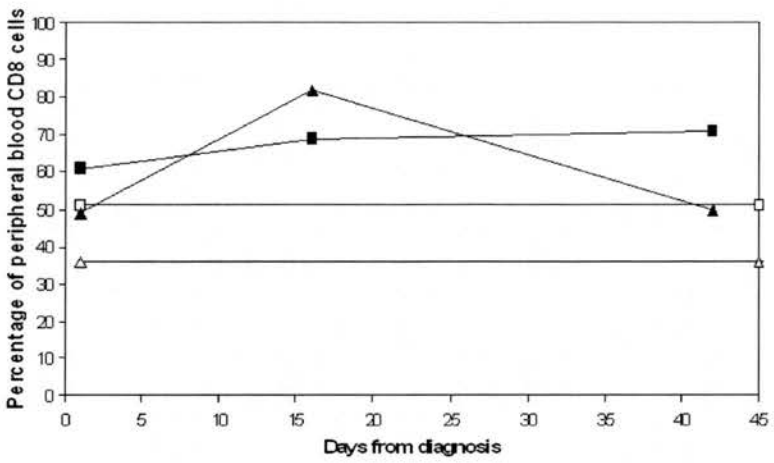


Figure 6-6 Expression of CD244 & SLAM is elevated during recovery from IM

The percentage of CD8 cells expressing CD244 (filled squares) and SLAM (filled triangles) are shown for case 9 (Table 5-6 page 107). Data are compared to median control values for CD244 (open squares), and SLAM (open triangles).

6.3 Extent of immune activation at diagnosis is significantly linked to severity of illness

We hypothesised that if the clinical symptoms of IM are caused by activated T cells rather than direct cytopathic effects of the virus, the extent of immune activation should predict clinical severity of IM. We therefore analysed the relationship between physical symptoms and function, and degree of immune activation in our cohort of 26 IM cases (Table 5-6 page 107).

The severity of sore throat was recorded in 26 cases and classified as either mild (14 subjects) or severe (12 subjects). The median percentage and number of lymphocytes expressing CD3, CD244/CD3, CD8, and CD244/CD8 at diagnosis were all significantly higher in the severe compared to the mild group ($p < 0.05$ Figure 6-7 page 123). The median total lymphocyte counts were higher in the severe compared to the mild group, however changes were not significant. Other immune parameters investigated at time of diagnosis, including CD4 + cells and T cell subsets expressing SLAM, were not significantly linked to severity of sore throat (Table 6-4 page122).

Of note, there was no significant difference in length of illness prior to diagnosis in those experiencing mild compared to severe sore throat.

Experience of fever was also recorded at the point in the illness at which the individual felt most unwell; 21 individuals experienced fever, the other 5 did not. There was a higher percentage of CD8+ T cells in the group with fever, median percentage of CD8+ T cells in the febrile group was 70% and in afebrile group was 44%, ($p<0.05$, Figure 6-8 page 124). For the other parameters measured, median expression was also higher in the febrile compared to the afebrile group. These included CD3, CD244/CD3, CD244/CD8 percentage expression, and CD3, CD244/CD3, CD8, and CD244/CD8 cell counts (Table 6-4 page 122). However median values were not significantly different between the 2 groups, possibly due to the small numbers of afebrile cases.

Lymphadenopathy was experienced by all cases, and so could not be linked to level of immune activation. Rash is another common symptom of IM, however the aetiology of the rash is unknown. Interestingly the percentage of the lymphocytes expressing CD3 was significantly lower in the 5 cases with rash, compared to the 20 cases without rash, (data unavailable in one case) with a median value of 60% compared to a median value of 79% respectively ($p<0.01$). None of the other immune parameters investigated were significantly different in those with compared to those without rash (Table 6-4 page 122).

Fatigue occurs in 90-100% of cases of IM. As discussed we aimed to make an objective measurement of fatigue, by recording whether individuals could leave home at the worst point in their illness. In our cohort 9 cases were house bound, and 17 were not. The percentage of CD8+ cells expressing CD244 was significantly higher in those who were housebound, ($p<0.05$) compared to those who were not, (median expression in the housebound group was 89%, and 78% in the group able to leave home). In addition, the median numbers of CD244/CD8 lymphocytes were higher in the housebound compared to the non housebound group (3.47×10^6 /ml compared to 2.87×10^6 /ml respectively), however these differences were not significant (Figure 6-9 page 125). Expression of the other parameters measured

including CD8, CD3 and CD244/CD3 subsets were not significantly different between the two groups (Table 6-4 page 122).

Table 6-4 Immune parameters and relationship to physical symptoms and function

Immune parameter	Sore throat	Fever	Leave home	Rash	University attendance
Lymphocyte count	NS	NS	NS	NS	NS
CD3 %	**	NS	NS	**	NS
CD3 count	*	NS	NS	NS	NS
CD8 %	**	*	NS	NS	NS
CD8 count	*	NS	NS	NS	NS
CD3CD244/CD3 %	*	NS	NS	NS	NS
CD3CD244 count	*	NS	NS	NS	NS
CD3SLAM/CD3 %	NS	NT	NS	NS	NT
CD3SLAM count	NS	NT	NS	NT	NT
CD8CD244/CD8 %	*	NS	*	NS	NS
CD8CD244 count	*	NS	NS	NS	NS
CD8SLAM/CD8 %	NS	NT	NS	NS	NT
CD8SLAM count	NS	NT	NS	NT	NT

** denotes differences between the medians of the groups are statistically significant, $p<0.01$

* denotes differences between the medians of the groups are statistically significant, $p<0.05$

NS denotes differences between the medians of the groups are not statistically significant, $p>0.05$

NT denotes differences between the groups were not tested The definition of the clinical events is described in the text.

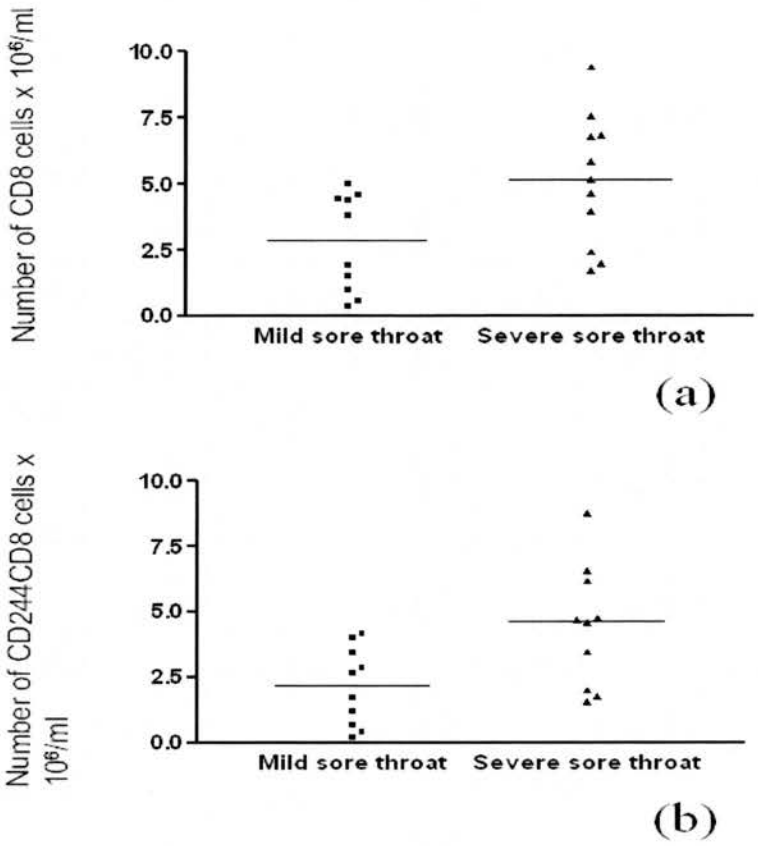


Figure 6-7 (a & b) CD8+ T cell lymphocytosis is higher in those with severe compared to mild sore throat.

(a) The median number of lymphocytes expressing CD8 was significantly higher in those with severe (n=11) compared to those with mild (n=10) sore throat ($p<0.05$). The median value for the severe group was 5.18 x 10^6 /ml for the mild group was 2.89 x 10^6 /ml. Bars show the median values.

(b) The median number of lymphocytes expressing CD244/CD8 was significantly higher in those with severe (n=10) compared to those with mild (n=10) sore throat ($p<0.05$). The median value for the severe group was 4.64 x 10^6 /ml and for the mild group was 2.20 x 10^6 /ml. Bars show the median values.

NB/lymphocyte counts were not available in all cases. All analysis refer to PBMC.

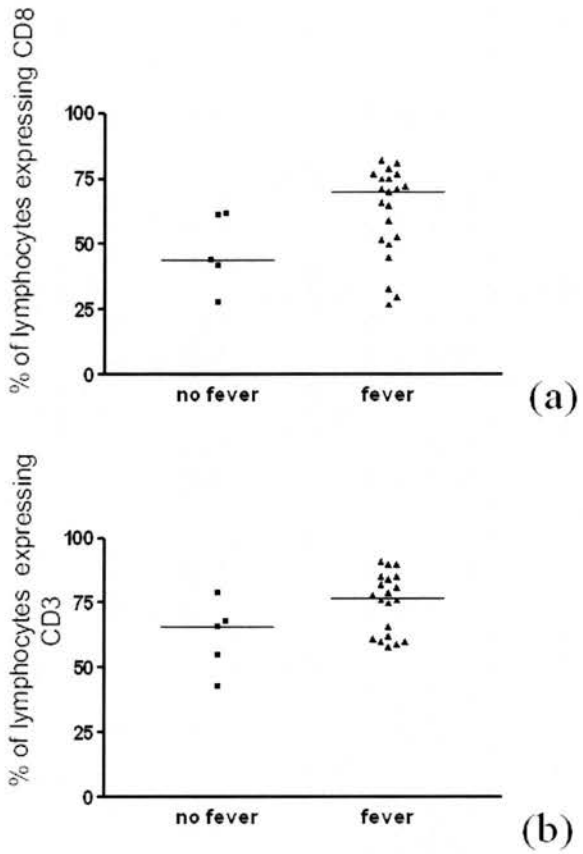


Figure 6-8 (a & b) CD8 T cell lymphocytosis is higher in cases of acute IM with fever than without fever.

(a) The median percentage of lymphocytes expressing CD8 was higher in those with fever (n=21) compared to those without fever (n=5). The median value for the fever group was 70% and for the afebrile group was 44%, the difference was significant ($P<0.05$).

(b) The median percentage of lymphocytes expressing CD3 was higher in those with fever (n=20) compared to those without fever (n=5). The median value for the febrile group was 77%, and for the afebrile group was 66%, however the difference between the 2 groups was not significant.

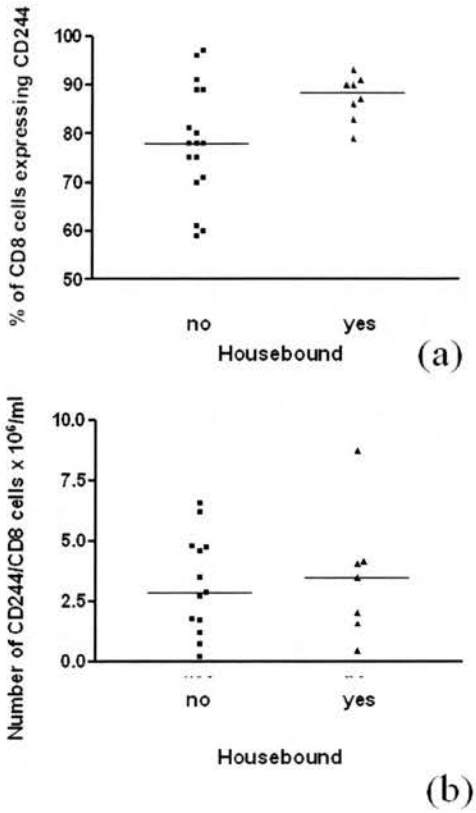


Figure 6-9 (a & b) CD8+ T cell lymphocytosis is higher in those unable compared to those able to leave home.

(a) The median percentage of CD8 cells expressing CD244 was higher in those who were house bound (n=8) compared to those who were not (n=17). The median value for the house bound group was 89% and for the group able to leave home was 78%, the differences between the 2 groups was significant ($P<0.05$).

(b) The median numbers of lymphocytes expressing CD244/CD8 was higher in those who were house bound (n=7) compared to those who were not (n=13). The median value for the house bound group was 3.47 x 10^6/ml and for the other group was 2.87 x 10^6 /ml however the difference between the 2 groups was not significant.

6.4 Immune activation at diagnosis of IM shows a strong positive correlation with viral load

Next we investigated the relationship between viral load and immune activation. Viral load was measured by PCR in the PBMC of 23 cases of IM at diagnosis (Table 5-6 page 107). The range of viral loads was 0 to 49249 EBV genomes per 10^6 PBMC. Increased viral load correlated directly with increased percentage of both CD3 ($p< 0.05$) and CD8 cells ($p<0.05$) in the peripheral blood at diagnosis of IM, and moreover increased viral load correlated directly with increased expression of CD244 on CD8 cells ($p<0.05$, Figure 6-10). Viral load did not correlate with CD4 counts or expression of CD244 on CD4 cells, or SLAM on either CD4 or CD8 subsets.

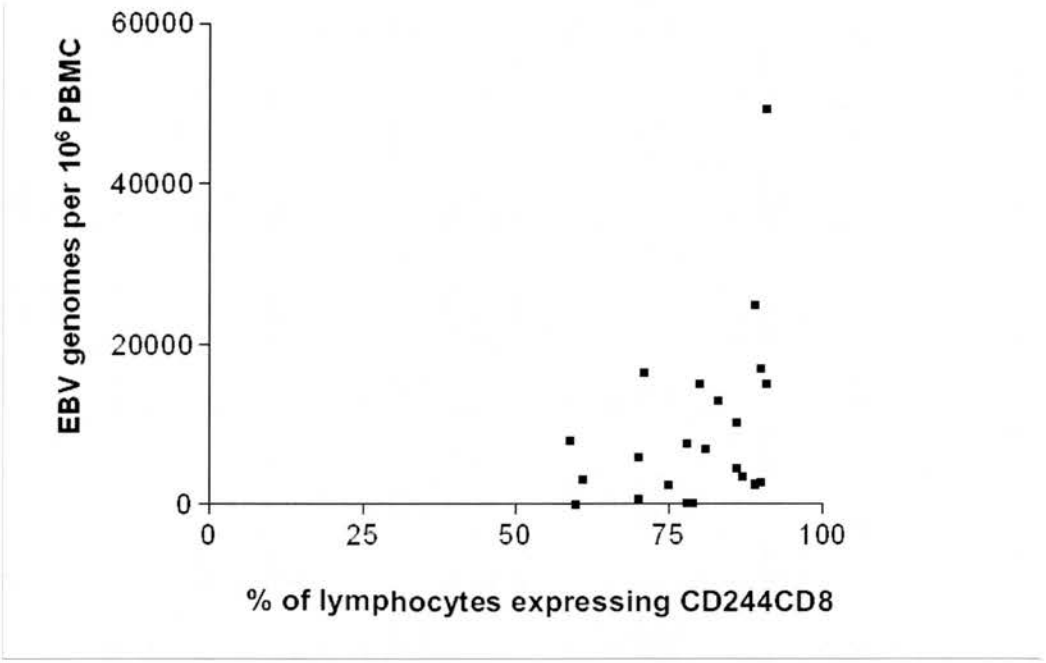


Figure 6-10 The percentage of lymphocytes expressing CD244/CD8 has a direct correlation with viral load. ($P<0.05$ and Pearson $r=0.41$).

In addition, median viral load was significantly higher in those with severe compared to mild sore throat ($p<0.05$) (Figure 6-11 page 127). The median viral load in those with severe throat was 6868 EBV genomes per 10^6 PBMC, and in those with mild sore throat was 2722 EBV genomes per 10^6 PBMC.

Figure 6-11 The viral load (EBV genomes per 10^6 PBMC) is higher in those with severe compared to mild sore throat

The difference between the median of the 2 groups was significant ($P<0.05$) and bars show median values.

6.5 Changes in CD244/CD8 are more pronounced in IM than primary VZV infection in adults.

In order to determine whether changes in the CD244/SLAM activation pathway are specific for primary EBV infection, we analysed CD244/SLAM in T cell subsets of patients with severe acute VZV infection. The median percentage expression of CD244 on CD3, CD4, CD8 subsets was raised in acute VZV cases; the range for CD3 cells was 24-63%, for CD4 cells was 5-23% and for CD8 cells was 53-69%. However, the median percentage expression was significantly higher in IM than VZV for both CD3 and CD8 subsets ($p<0.05$, Figure 6-12 page 128). There was an indication of an increased percentage expression of SLAM on CD3, CD4, CD8 subsets in VZV cases, but levels of expression were not significantly different from either control subjects or IM cases. Thus, CD244 is significantly upregulated on T lymphocytes during acute IM and VZV infection on both CD3 and CD8 subsets, but activation is more marked during IM than VZV.

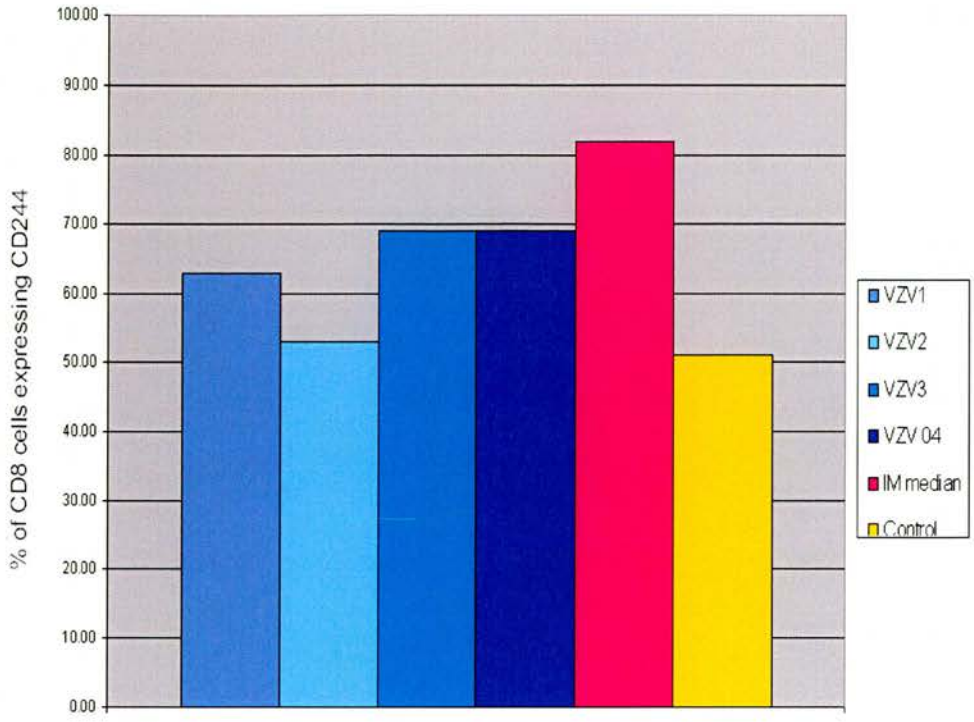


Figure 6-12 The median percentage of CD8 cells expressing CD244 is higher in IM than in VZV infection

The percentage of CD8 cells expressing CD244 is shown for the 4 cases of VZV and the median counts for IM cases and controls are also shown. The median percentage of CD8 cells expressing CD244 was higher in IM than in VZV ($p<0.05$); the median expression in IM was 82% and in VZV was 63%.

7 Expression of SAP in Infectious Mononucleosis

In Chapter 6 (page 109) we showed that significant up-regulation of both SLAM and CD244 on lymphocytes occurs during acute IM. Therefore we decided to monitor the expression of SAP, the intracellular regulator of this lymphocyte activation pathway, during acute IM. Immunohistochemistry was used to assess the expression of SAP in a variety of cells and tissues both from the IM cohort and healthy controls. As detailed in the methods, intracellular flow cytometry could not be used to monitor SAP expression due to technical difficulties (Section 3.8.3 page 80).

7.1 Optimisation of single colour immunohistochemistry.

The experiments were undertaken as detailed in the methods (Section 3.9 page 81), using DAB single staining, however the following optimisation steps were carried out.

7.1.1 Cytospin Preparation

Optimal results were obtained using fresh PBMCs to make cytospins. However reasonable results could be obtained using frozen cells if dead cell numbers were minimized by rapid thawing of the cells. If numbers of dead cells were high, at approximately greater than 10%, increased background staining occurred. We also found long term storage of the cytospins, even at -20^0 C, led to poor staining with loss of cell integrity, and optimal results were acquired within 3 months of making the slides.

7.1.2 Antibody Titration

Polyclonal anti-SAP antibody was available commercially, either derived from rabbit or goat. Both types of antibody were diluted at 1:10, 1:20 and 1:50 in DAKO antibody diluent, and staining of control sections (PHA stimulated PBMC) was carried out. Problems with excessive background staining were encountered; however a 1:20 dilution of goat antibody gave the best results and was used throughout (Table 7-1 page 130).

Table 7-1 Results of antibody titration

Dilution of antibody in DAKO diluent.	Goat anti SAP antibody		Rabbit anti SAP antibody	
	Background staining	Positive staining	Background staining	Positive staining
1:10	++	++	+++	++
1:20	+/-	++	++	+
1:50	-	+	+	+

++ = good signal + = moderate signal - undetectable signal

7.2 Expression of SAP in peripheral blood lymphocytes during acute IM and in controls

7.2.1 Expression of SAP is elevated during acute IM

Expression of SAP in PBMC was analysed by immunohistochemistry in 16 cases of acute IM and in 17 healthy controls. A negative control slide, without primary antibody, was included in each experiment, and always gave negative results. A positive control of either PHA blasts or a known positive sample was used during optimization experiments.

At the time of diagnosis of IM, the number of PBMC expressing SAP was high with 54-100% of cells expressing SAP, compared to 2–74% of PBMC from controls (Figure 7-1 page 132). The median values were significantly different between controls and cases: the median value for cases was 85% compared to the median values for controls of 33% ($p<0.001$, Table 7-2 page 131).

7.2.2 SAP expression remains elevated during recovery from acute IM

Expression of SAP in PBMC was analysed in 8 cases over the course of IM, at time points between 11-139 days following diagnosis (median 24 days). Although SAP expression fell over the course of the illness, ranging from 41-73 % of PBMC, values remained significantly raised compared to controls ($p<0.01$, Table 7-2 page 131 and Figure 7-2 page 132).

Table 7-2 Expression of SAP in PBMC

Case or control Group	Number of cases	% of PBMC expressing SAP
At diagnosis of IM	16	Range 54-100% Median 85%***
During recovery from IM	8	Range 41-73% Median 54 %*
Healthy controls	17	Range 2-74% Median 33%

*** median values were significantly different to control values ($p<0.001$)

* median values were significantly different to control values ($p<0.05$)

Figure 7.1 PBMC from 4 acute IM cases showing positive staining for SAP (stained brown). The magnification is shown for each image.

a x 600

b x 400

c x 400

d x 1000

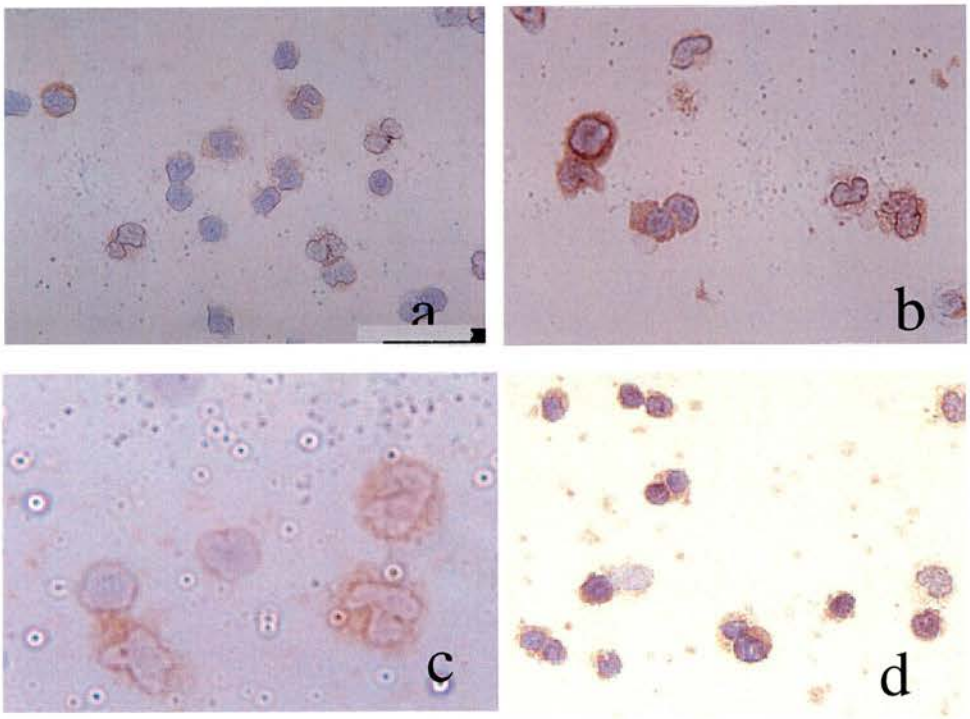


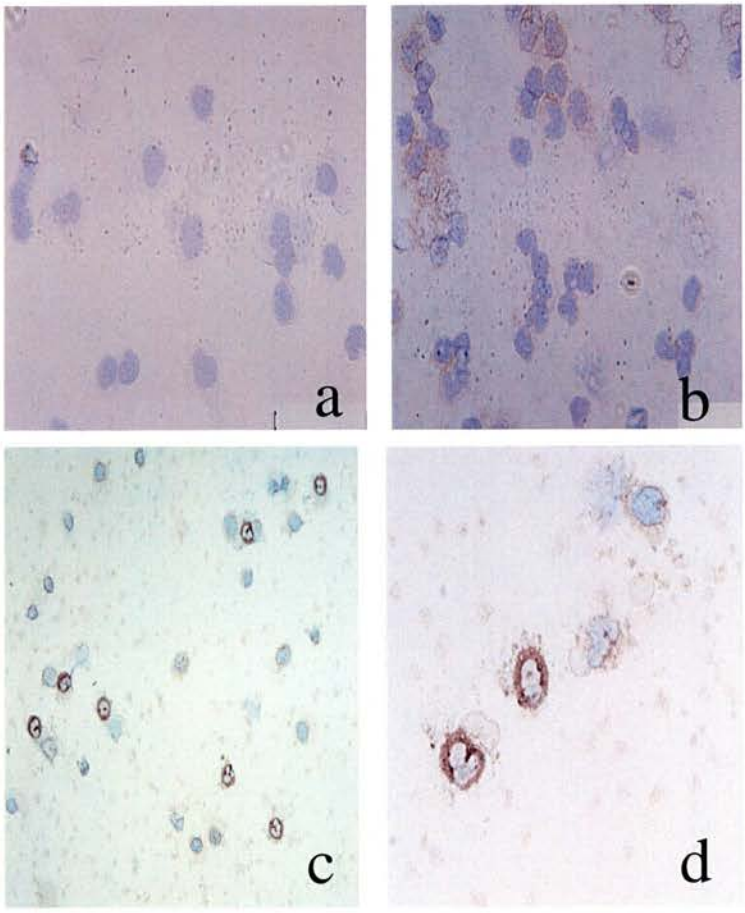
Figure 7.2 PBMC from acute IM cases and controls showing staining for SAP (stained brown). The magnification is shown for each image.

a. Healthy control with no antibody (negative control) x 400

b Healthy control x 400

c IM follow up (day 21) x 200

d As image c, x 1000



7.2.3 Relationship between expression of SAP and expression of CD8, CD244/CD8 and SLAM/CD8 on lymphocytes in acute IM

In 9 control subjects we did not find a significant relationship between the percentage of cells expressing SAP and either the percentage of CD8 cells, or the percentage of CD8 cells expressing either CD244 or SLAM cells in the peripheral blood. However in the 10 cases analysed at diagnosis of acute IM, there was a significant correlation between the percentage of CD8 cells expressing CD244 and the percentage of SAP positive cells in the peripheral blood (Pearson r =0.71, $p<0.05$ and Figure 7-3 page 134). However, as in the control data, no relationship was found between SAP expression and the percentage of cells expressing CD8 or CD8/SLAM.

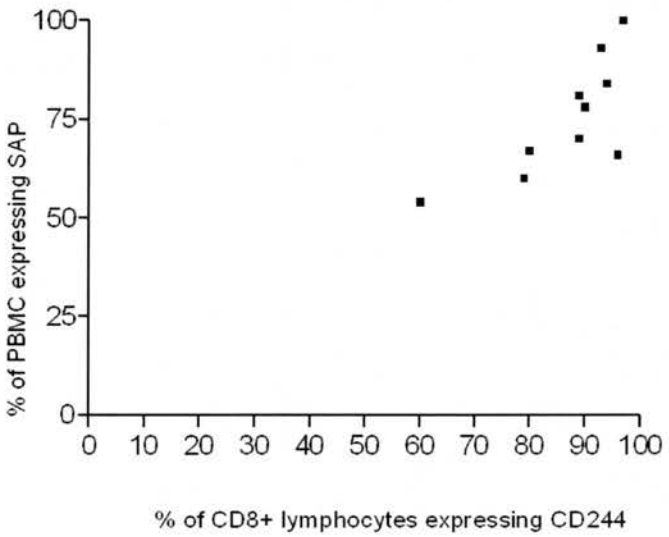


Figure 7-3 The percentage of CD8+ lymphocytes expressing CD244 correlates with the percentage of PBMC expressing SAP.

There is a significant correlation between the two parameters (Pearson r=0.71, $p<0.05$).

7.2.4 Expression of SAP in tissue from acute IM

Tissue (colon) was available from one case of fulminant IM, case 10 in the XLP mutation study, (Table 4-1 page 89). Routine diagnostic immunohistochemistry showed a lymphocytic infiltration within the mucosa, with both B cells (CD20+) and T (CD3+) cells present. Within the lymphocyte infiltration, approximately 30-50% of the cells were noted to express EBNA-2 protein (FIgure 7.4 page 136), though

double staining for lymphocyte subtype within the EBNA-2 population was not undertaken. There was also a large number of EBER positive cells (Figure 7.4 page 136) and SAP was widely distributed (Figure 7.5 page 137). In addition occasional cells were found to express LMP1.

Figure 7.4 show expression of EBV antigens in a case of severe IM, (case 10, *chapter 4*). (a) shows EBER staining (brown) x 200 and (b). Shows EBNA2 staining (brown) x 200. Arrows indicate a typical positive cell.

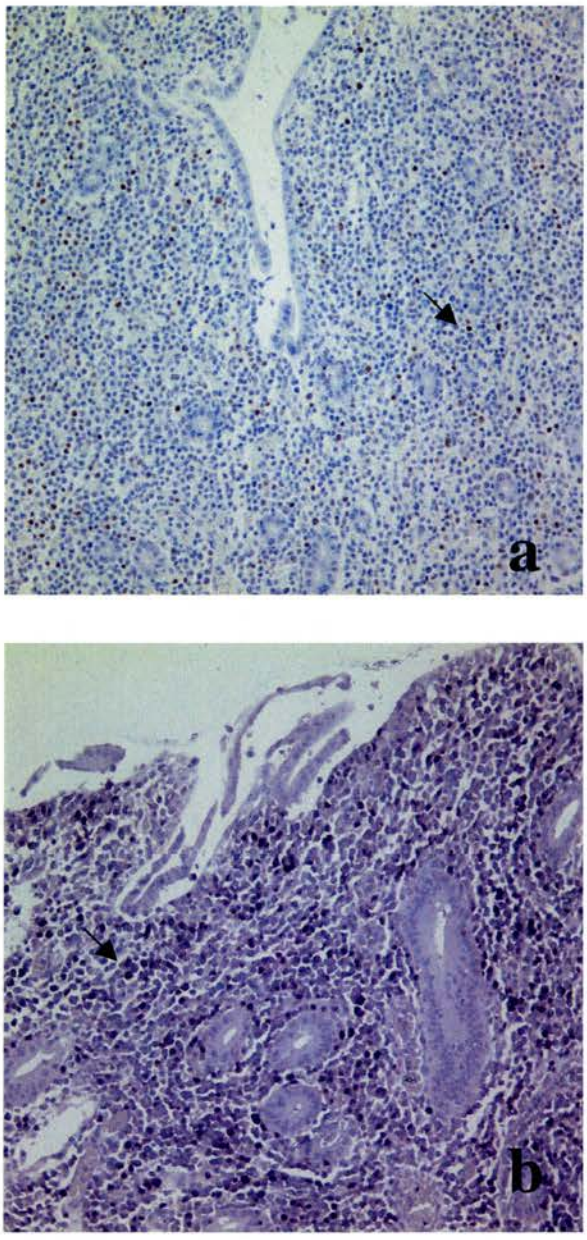
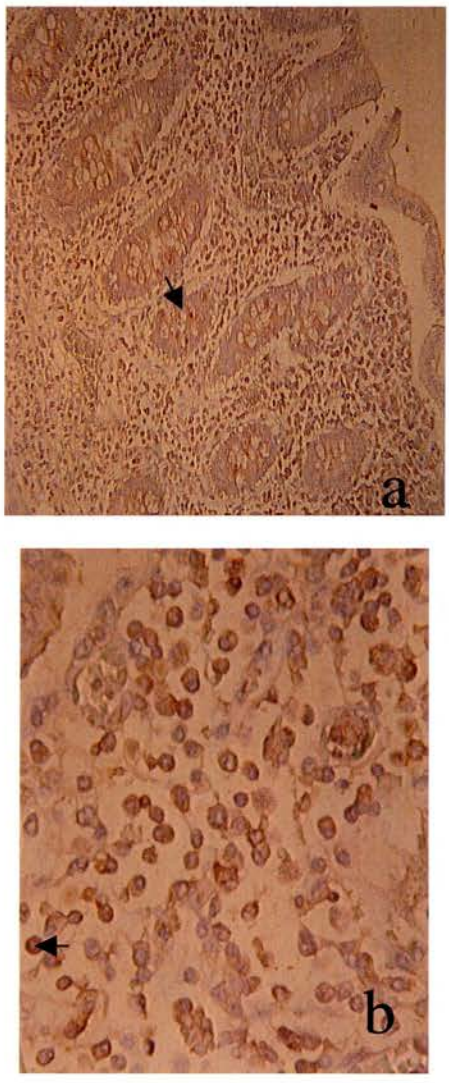


Figure 7.5 shows expression of SAP in the colon in severe IM (case 10, ***chapter 4***). Magnifications are shown for each image (a) x 200 and (b) x400. Arrows show typical positive cell.



7.3 Dual expression of interferon gamma and SAP in peripheral blood cells from acute IM

Activated CD8+ T cells are able to release TH1 type cytokines and this process is thought to be critical to the pathogenesis of acute IM. We therefore investigated whether cells expressing SAP also expressed IFN γ during acute IM.

Dual staining for INF γ and SAP was carried out on fixed PBMC from 5 cases of acute IM, 5 healthy controls, 3 cases during recovery from IM, and 5 positive controls (PHA blasts) (Section 3.9.5 page 82).

In 4/5 cases of acute IM examined, expression of dual positive cells was high, in 1 case expression was low (the definition of high and low staining is described (Section 3.9.5 page 82)). Of note is that all cells that expressed INF γ also expressed SAP. In 2/3 cases studied during recovery from IM, no dual positive cells were identified, however in 1 case low numbers of dual positive cells were identified. In 5 healthy controls no dual positive cells were identified. Dual expression of INF γ and SAP was high in 5 out of 5 cases of PHA stimulated control cells; the positive control (Figure 7.6 page 139).

We therefore confirmed that cells expressing SAP during acute IM, co-express INF γ, and therefore are able to contribute to the pathogenesis of acute IM.

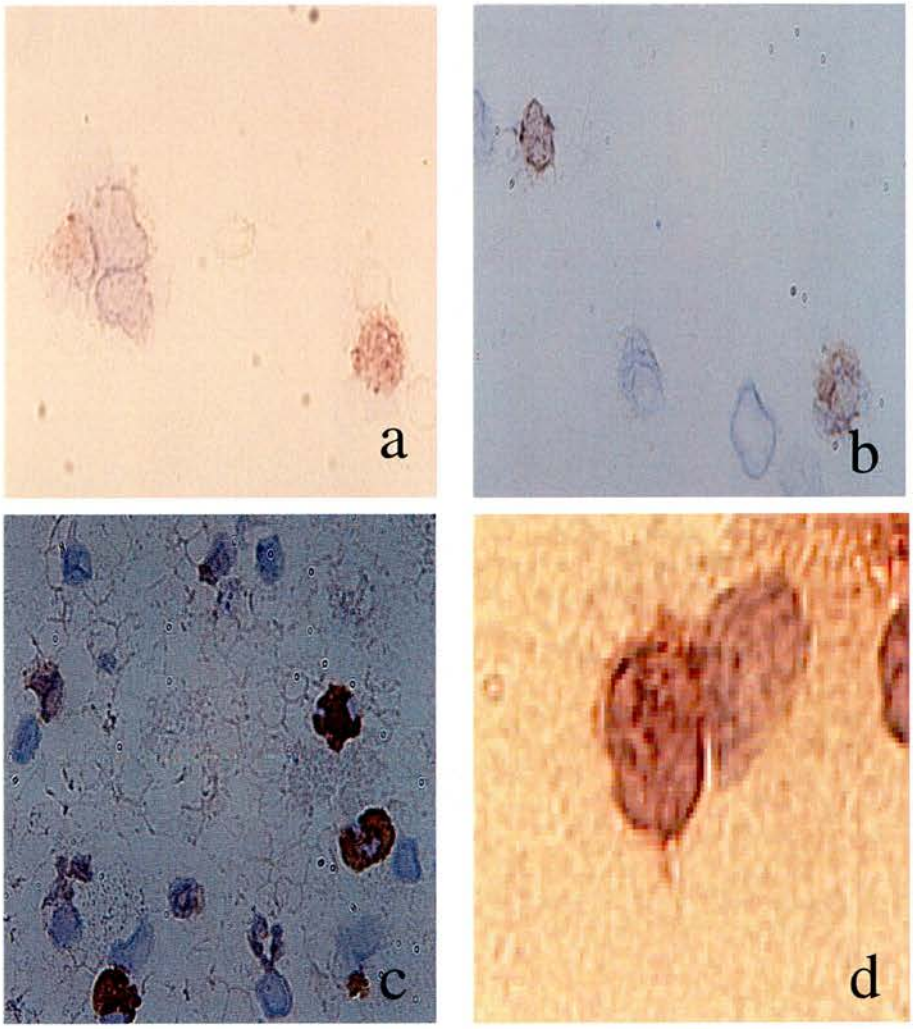
Figure 7.6 PBMC from control and 3 acute IM cases showing staining for SAP (stained blue) and INF γ (stained brown). The magnification is shown for each image.

a. PHA blasts x 1000

b Acute IM x 400

c Acute IM x 400

d Acute IM x 1000



8 Immune response to primary Epstein-Barr virus infection: a critical role for natural killer cells

The role of antigen specific CD3+ CD8+ cytotoxic T cells in the control of primary EBV infection is well established (Section 2.6.8.1 page 34). However, time is required for the antigen specific immune response to develop and expand. In contrast innate immune responses, such as NK cells, are thought to be vital early in the infection process, but data are lacking on the activity of NK cells during viral infection in humans. The scale, phenotype and function of the NK cell response during IM are described in this chapter.

8.1 NK cell numbers are significantly elevated at diagnosis of IM

NK cell numbers were analysed by flow cytometry at diagnosis, and during recovery from IM, and compared to data from healthy controls. The NK cell subset was defined as cells which fell into the lymphocyte gate, which expressed CD56 but did not express CD3. Of note, all cells which expressed CD56 also expressed CD244. NKT cells were defined by expression of CD56 and CD3 (Figure 8-1 page 140).

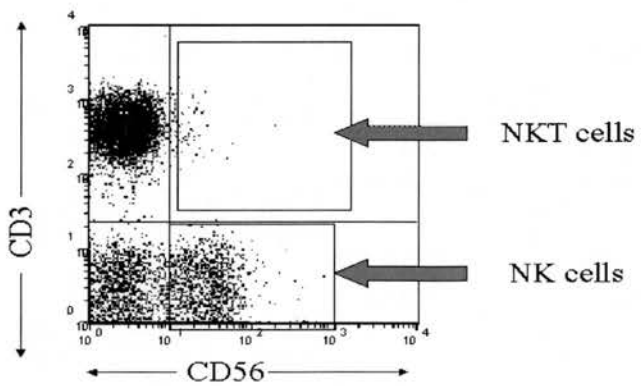


Figure 8-1 Identification of NK cells by flow cytometry

The flow plot shows how NK and NKT cell subsets were identified in the lymphocyte population. NK cells were defined as expressing CD56 but not CD3, NKT cells were defined as expressing CD3 and CD56.

In patients with acute IM, (N=26 with lymphocyte counts available in 21 cases) NK cells were significantly elevated in the peripheral blood at diagnosis compared to controls (N=23, with lymphocyte counts available in 17 cases). Median counts for IM cases were 0.87 x 10^6 per ml, with 5-38 % of lymphocytes expressing a NK cell phenotype. This compared to healthy controls where median counts were 0.14 x 10^6 per ml with 1-19% of lymphocytes showing an NK cell phenotype, (p=<0.01, Table 8-1 page 141 and Figure 8-2 page 143).

Table 8-1 NK cell counts in PBMC from IM cases and controls

IM Case (n=26)	NK cell numbers x 10^6/ml	NK cell % of lymphocytes	Controls (n=23)	NK cell numbers x 10^6/ml	NK cell % of lymphocytes
1	Na	11	1	0.14	11
2	0.9	38	2	0.32	15
3	0.56	21	3	0.07	5
4	0.42	8	4	0.22	17
5	0.9	15	5	0.38	18
6	2.17	20	6	0.06	5
7	1.31	15	7	0.12	8
8	2.0	34	8	0.05	3
9	1.57	22	9	0.15	12
10	2.1	28	10	0.12	10
11	Na	29	11	0.41	14
12	Na	17	12	0.05	5
13	0.87	20	13	0.03	6
14	2.5	20	14	0.35	15

15	1.01	12	15	0.22	11
16	1.1	13	16	0.09	5
17	0.64	10	17	0.22	10
18	0.1	8	18	Na	6
19	0.5	5	19	Na	7
20	0.55	6	20	Na	11
21	0.54	16	21	Na	1
22	0.7	19	22	Na	7
23	Na	12	23	Na	13
24	Na	16			
25	0.44	17			
26	0.15	7			
Median	0.87	17%	**Median**	0.14	10%
Range	0.10-2.50	5-38%	**Range**	0.03-0.41	1-19%

Na - data not available

In a subgroup of 11 cases the proportion of lymphocytes expressing NKT cell markers, was also investigated. There was no significant expansion of NKT cells in acute IM, with a median of 1% of lymphocytes expressing CD3+CD56+CD244+, compared to a control median of 2%.

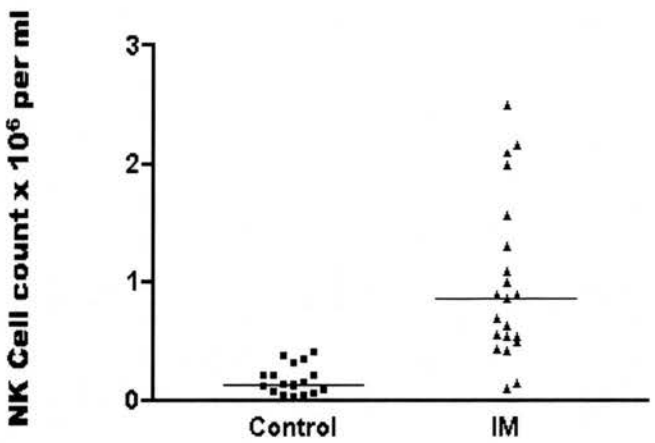


Figure 8-2 NK cells are raised in the peripheral blood at diagnosis of acute IM.

A scatter plot of NK cell counts x 10^6 per ml for controls (N=17) (filled squares) and cases (N = 21) (filled triangles) is shown. Median counts are shown by bars, and for cases was 0.87 x 10^6 per ml and for controls was 0.14 x 10^6 per ml. The difference between the median values for the 2 groups was statistically significant ($p<0.01$).

8.2 NK cells remain elevated during recovery from IM

In order to relate the clinical symptoms of IM to changes in NK cell numbers a study of NK cell numbers in the peripheral blood, was carried out during the course of IM on a group of 6 patients (cases 1,4, 9, 10, 11 and 12 in Table 5-6 page 107).

As documented, NK cell counts were elevated at diagnosis. At a subsequent review at the time point closest to 1 month following diagnosis, NK cell counts had fallen (range 0.14-0.50 x 10^6 per ml) but remained elevated compared to controls ($p<0.01$ Figure 8.3 page 144). However, at this time point both the total lymphocyte counts and CD8+T cell counts for cases had fallen and were not significantly different from controls (Table 6-1 page 110): median lymphocyte counts for 6 cases of IM were 1.82 x 10^6 per ml and 1.35 x 10^6 per ml for controls, with CD8 positive lymphocytes 0.44 x 10^6 per ml for cases and 0.48 x 10^6 per ml for controls. 5 out of the 6 cases studied at this time point continued to be unwell, with symptoms such as enlarged glands and fatigue (Figure 8-4 page 145). By 6 months after diagnosis NK cell counts in all cases had fallen to within the range for controls (0.03-0.41 x 10^6 per ml), and median counts were not significantly different to controls.

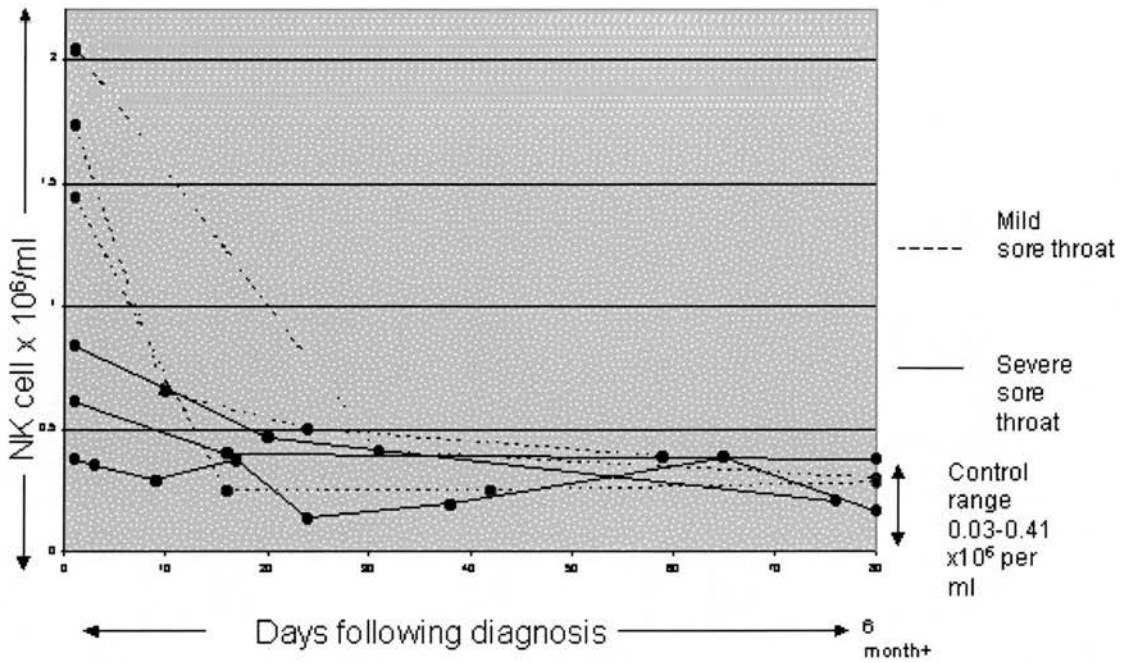


Figure 8-3 NK cell counts are raised in the first month following diagnosis of IM

A line graph is shown of NK cell counts from 6 cases of IM at diagnosis to 6 months post diagnosis. Cases with severe sore throat are shown by an unbroken line and cases with mild sore throat are shown by a broken line (See section 8.4 page 159).

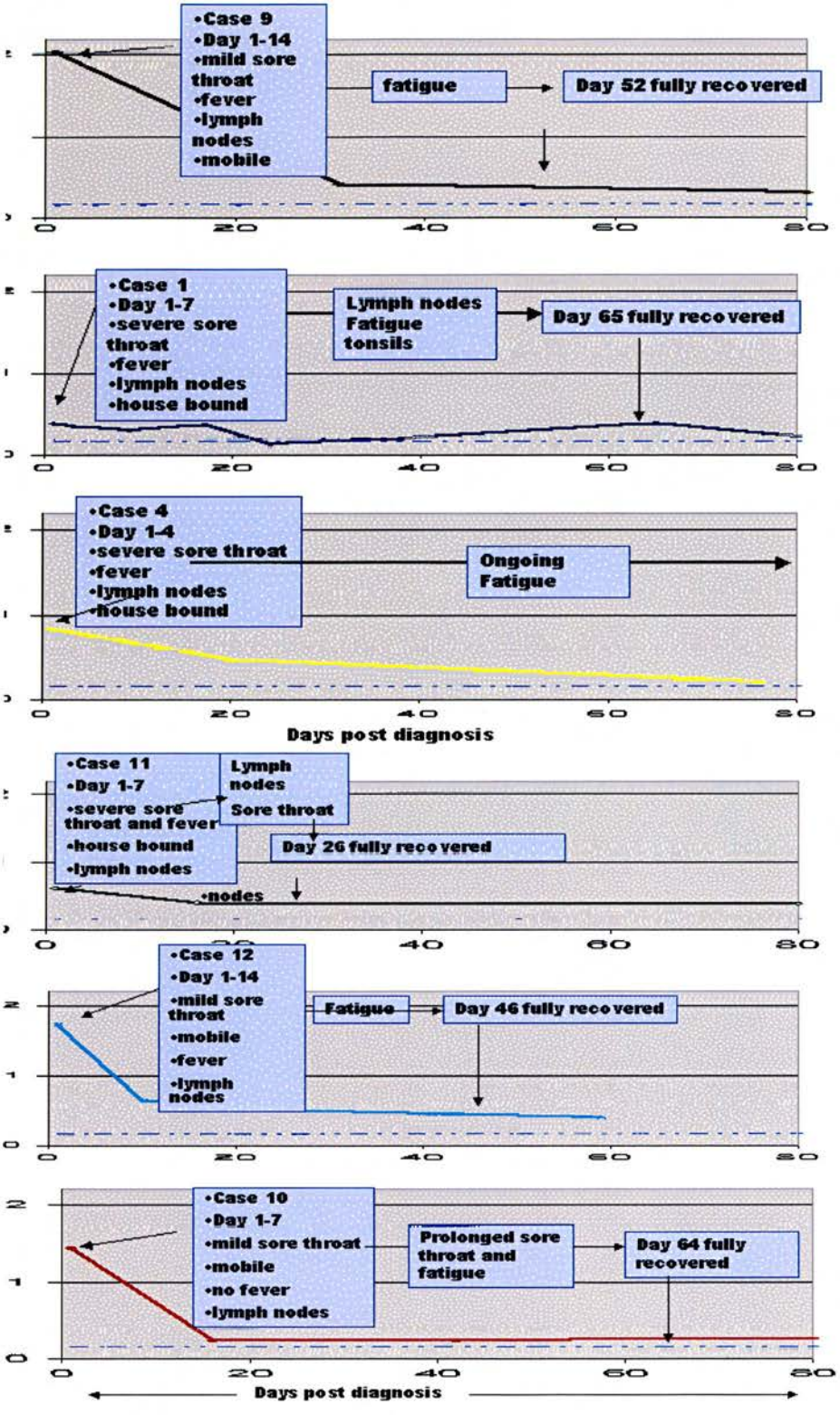
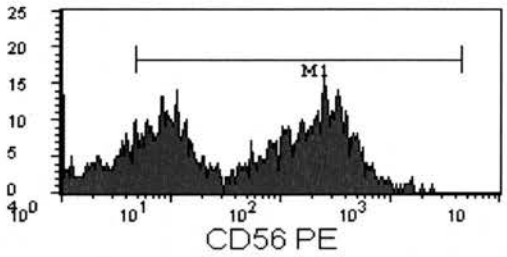


Figure 8-4 NK cell counts and key clinical features in 6 cases of IM

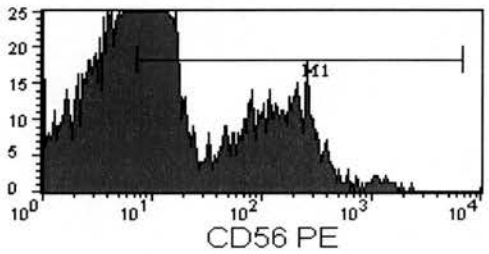
8.3 The CD56^{bright} cell population is expanded in acute IM

As NK cell numbers were shown to be elevated in acute IM, changes in cell phenotype, which may contribute to the pathogenesis of IM were investigated. CD56^{bright} cells are important producers of regulatory cytokines and may provide a link between the innate NK cell and acquired CD8 cell response to infection (Cooper et al., 2001a).

A number of investigators have noted that when analysed by flow cytometry, expression of CD56 on NK cells can be categorised as bright or dull. Approximately 90% of resting cells express low levels of CD56, and are defined as CD56^{dull} cells and 10% of resting cells express high levels of CD56, and are defined as CD56^{bright} cells (Lanier et al., 1986). However, there is no absolute classification of a CD56^{bright} cell, and the fluorescent intensity recorded by the cytometer will depend on certain machine parameters as well as the fluorescence emitted by the cell. Therefore CD56 expression was analysed both as a single parameter, median fluorescence intensity (MFI) (Figure 8.5 page 147), and also by recording the percentage of the total NK cell population which showed high levels of CD56 expression. In order to enable comparison between different experiments, the voltages, gates and compensation settings on the flow cytometer were not changed between experiments.



MFI =159



MFI = 29

Figure 8-5 MFI of CD56 on NK cells is raised in IM compared to controls

The histograms show the method of calculation of MFI expression of CD56 on NK cells from a subject with acute IM (top plot) and a control subject (bottom plot).

The analyses of intensity of CD56 expression as a single parameter was carried out first, and a statistically increased MFI of CD56 was found on NK cells from IM patients (n= 8) compared to controls (n=9) ($p<0.001$). MFI for cases was 125 and for controls was 25. Next the proportion of NK cells in IM expressing a $CD56^{bright}$ cell phenotype was analysed, and again this was found to be significantly raised (median of 17% compared to a control median of 6%) ($p< 0.01$, Figure 8.6 page 148 and Figure 8-7 page 149). Unlike studies on cells from healthy donors or cultured NK cells (Cooper et al., 2001b), a proportion of $CD56^{bright}$ cells were also found to concurrently express CD16 (Figure 8-7 page 149). It is therefore possible that this population is not an expansion of the $CD56^{bright}$ cell population, but a population of NK cells which show increased expression of CD56 during acute IM. CD244 is constituently expressed on NK cells (Nakajima et al., 1999), and there was no difference in intensity of expression of CD244 on NK cells from IM cases than from controls (MFI 242 in cases and MFI 223 in controls).

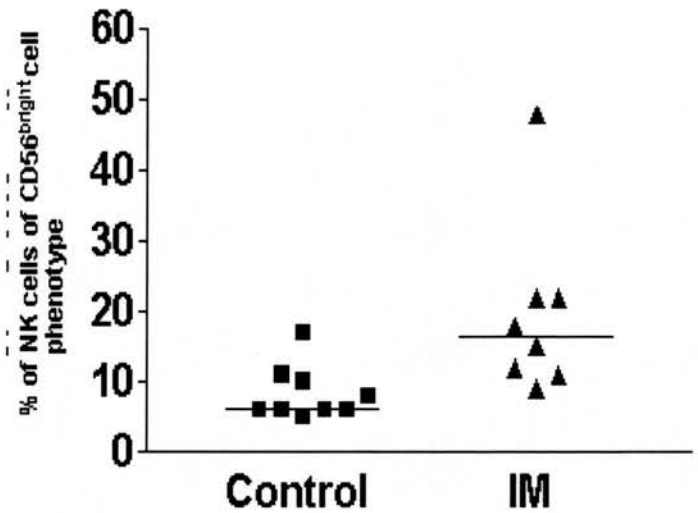


Figure 8-6 The CD56^{bright} cell population is expanded in acute IM.

A Scatter plot of the percentage of NK cells of CD56^{bright} cell phenotype for IM cases (n=8) and controls (n=9) is shown. Bars show median values of 17% for cases (filled triangles) compared to 6% for controls (filled squares). The difference between the median values for the 2 groups was statistically significant ($p<0.01$).

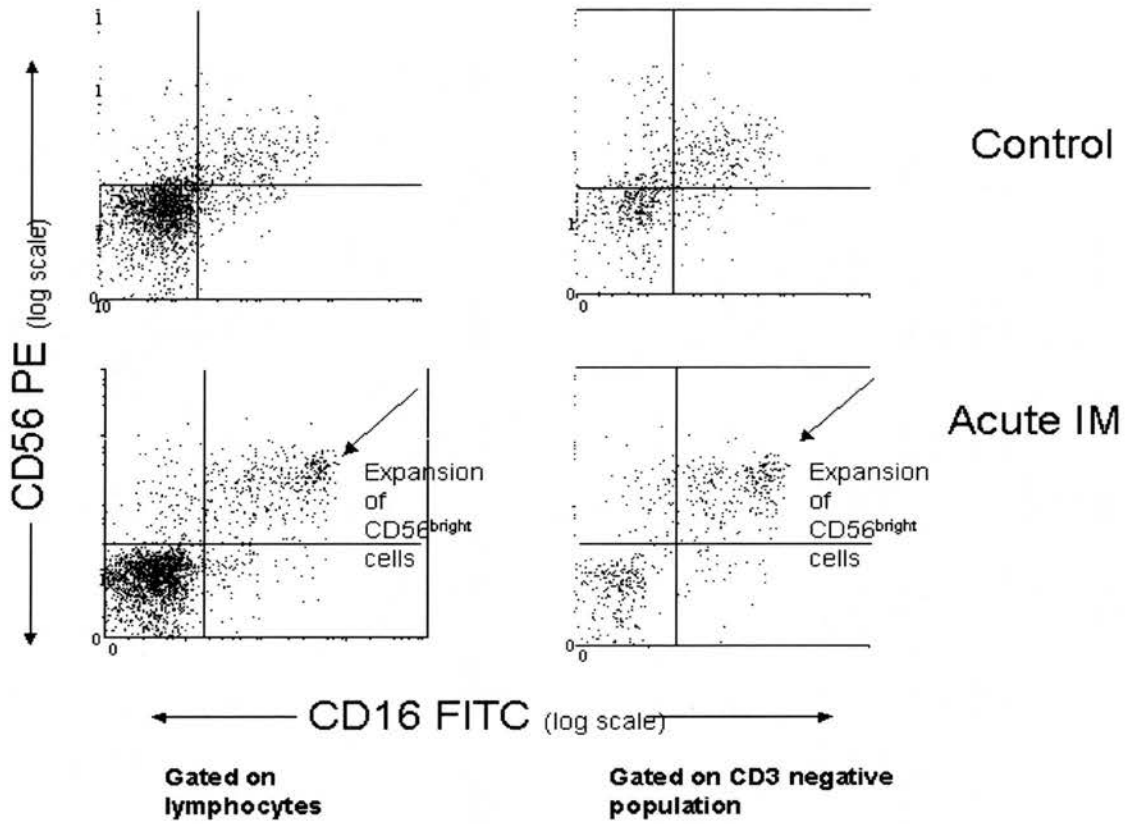


Figure 8-7 The FACS plot shows the expansion of a CD56^{bright} cell population in a typical case of IM compared to a healthy control

The 2 plots on the left are gated on all lymphocytes and the 2 plots on the right are gated solely on CD3 negative lymphocytes. The arrows identify the NK cell population which express high levels of CD56 which is expanded in acute IM. A comparison of the ability of NK cells from cases of IM and controls to kill an EBV infected cell line

As we had identified a change in NK cell phenotype during acute IM, we next investigated if this change in phenotype was associated with a change in NK cell function. NK cells are able to kill cell lines latently infected with EBV (LCLs) (Blazar et al., 1980), and therefore the ability to kill EBV infected cell lines was compared between NK cells from acute IM cases and controls.

8.3.1 Optimization of methods

8.3.1.1 NK cell separation by magnetic beads results in pure NK cell population.

NK cell separation was carried out using magnetic beads as described in the methods (Section 3.10 page 84). The cell purity of the resulting NK cell population was checked by standard FACS analysis on 5 occasions. The NK cell population was stained using antibodies to CD56 and CD3, and found to routinely contain less than 0.5 percent CD3 positive cells on all occasions (Figure 8-8 page 151). Furthermore, the population did not contain any B cells (CD19+), monocytes (CD14+) or CD4 + T cells. Following separation a substantial number of cells were present in the NK cell fraction which could not be identified, and were negative for B, T and NK cell markers. It was not possible to identify their phenotype further, however it is unlikely they would alter the subsequent cytotoxicity assay as they did not have a T cell phenotype. A reduction in the number of cells was found following addition of lysis buffer, suggesting a proportion may be red cells or red cell aggregates.

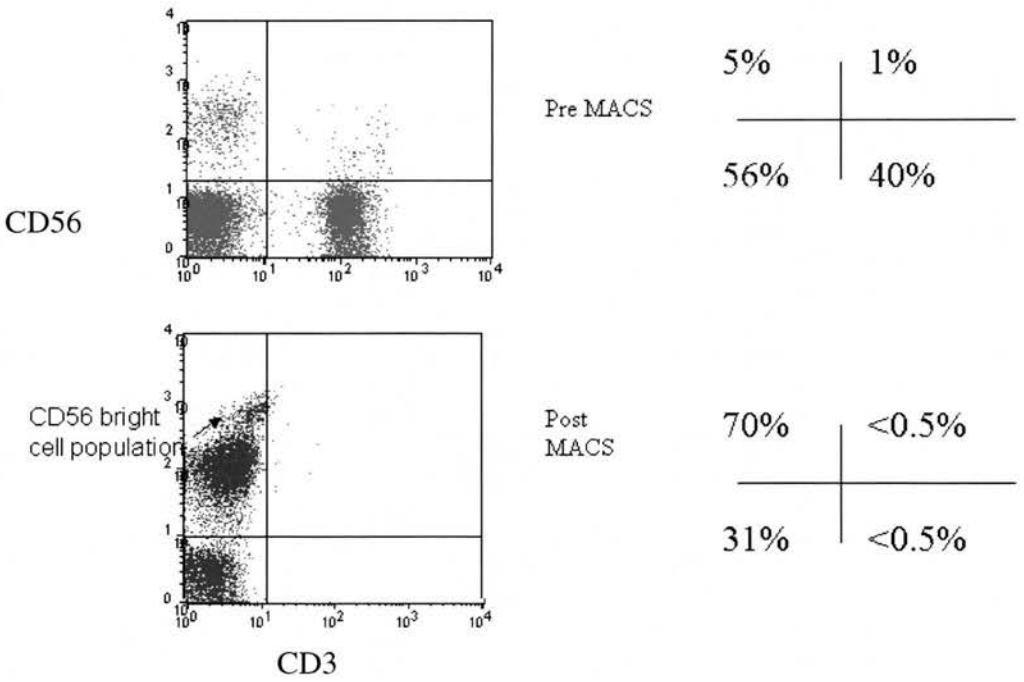


Figure 8-8 NK cell populations are pure post MACS bead separation.

The top plot shows the lymphocyte population pre MACS bead separation and the bottom plot shows the population post separation. The figures on the right show the percentage of cells in each quadrant. The cells were stained with CD3FITC (X axis) and CD56PE (Y axis).

8.3.1.2 NK cell cytotoxicty assay

8.3.1.2.1 Method 1- NK cell killing of autologous LCL

In the first method used the aim was to compare NK cell cytotoxicity from IM cases and healthy controls against an autologous LCL. It was decided to use NK cells directly ex-vivo, rather than clone the cells, as cell culture could lead to changes in cell phenotype and function. Therefore fresh PBMC were used to set up an LCL, and the rest of the sample was frozen within 48 hours of the blood being taken. The NK cell population was separated from the frozen stored PBMC, once the LCL was established. In the preliminary experiments using healthy controls, the subjects were re-bled to obtain fresh NK cells at the time of the cytotoxicity assay.

LCLs were grown from 8 healthy controls and 6 cases of IM. The cytotoxicity assay was carried out as described (Section 3.11 page 85), and on all occasions the target LCL and NK cells were autologous. A positive control for NK cell killing, the cell line K562, was included in all experiments.

8.3.1.2.2 LCLs grown for10 days are not killed by NK cells

In 2 control experiments the LCL was used as the target cell line 10 days after EBV infection and culture. However, at this stage of culture the NK cells failed to lyse the target LCL, despite effective lysis of K562 cells. In the same 2 donors, at week 7 of LCL culture, substantial killing of both LCL and K562 occurred.

Table 8-2 NK cell killing of LCL at 10 days and 7 weeks of tissue culture

	% cytotoxicity of K562			% cytotoxicity against LCL at 10 days of culture		
	20:1	10:1	5:1	20:1	10:1	5:1
Control 1	25	17	11	0	0	0

Control 2	92	100	100	0	0	0

	% cytotoxicity of K562			% cytotoxicity against LCL at 7 weeks of culture		
	20:1	10:1	5:1	20:1	10:1	5:1
Control 1	22	15	23	9	5	3
Control 2	40	41	8	39	55	31

The results are shown above in (Table 8-2 page 151).Therefore in all further experiments the LCL was grown for at least 5 weeks.

8.3.1.2.3 NK cells separated from cyropreserved PBMC have impaired killing capacity immediately after defrosting

When LCL growth was established, viable PBMC were rapidly defrosted and the NK cell fraction was separated and used immediately as effector cells in a standard cytotoxicity assay.

In the first experiment, NK cells were separated from 1 acute case of IM and 1 healthy control. The cytotoxicity assay was carried out immediately after separation of NK cells, and no killing of either LCL or K562 occurred (Table 8-3 page 153).

In a further 2 control experiments the NK cells were placed in an incubator for 2 hours at 37^0 C, before use in the cytotoxicity assay. A 2 hour incubation was chosen as this allowed the cytotoxicity assay to be completed within 1 day.

A slight improvement in killing occurred with low percentage killing of K562; however at all ratios killing of less than 10% was recorded. In contrast the median killing of K562 by fresh NK cells from controls was 40% at ratio of 20:1, 31% at 10:1, and 22% at 5:1 (Table 8-4 page 154) for details of individual control experiments), and no killing of LCL occurred. In comparison, in a further 2 control cases in which NK cells were separated and suspended in medium, and then left at 37^0 C overnight, levels of killing comparable to those obtained from fresh NK cells occurred (Table 8-3 page 153). Separation of NK cells from fresh PBMC and storage at 4^0 C overnight did not affect their ability to lyse target cells.

8.3.1.2.4 NK cells from IM cases stored for 12 hours clump

Following the above experiments, NK cells separated from frozen PBMC were stored overnight at 37^0 C. However NK cells from IM cases tended to form cell clumps overnight, and despite use of DNAase it was not possible to obtain enough NK cells for meaningful cytotoxicity assays.

Table 8-3 NK cell killing capacity of recently thawed and incubated cells

		Percentage killing of target cells					
NK cells separated from *frozen* PBMC		Ratio of NK cell to K562			Ratio of NK cell to LCL (cultured 5-7 weeks)		
		20:1	10:1	5:1	20:1	10:1	5:1
NK cells used immediately after separation	IM Donor 1	0	0	0	0	0	0
	IM Donor 2	0	0	0	0	0	0
NK cells used after 2 hours at 37^0 C	Control	6	9	8	0	0	0
	Control	7	8	4	0	0	0
NK cells used after 14 hours at 37^0 C	Control	NA	34	20	NA	NA	NA
	Control	NA	33	34	NA	NA	NA
NK cells separated from *fresh* PBMC	Control	NA	30	18	NA	17	2
	Control	40	41	8	39	55	31

NA denotes data unavailable.

Table 8-4 Percentage of Killing of LCL and K562 cell lines by autologous NK cells

Controls		% Killing of target cell					
		Ratio of NK cell to K562			Ratio of NK cell to LCL		
		20:1	10:1	5:1	20:1	10:1	5:1
NK cells from fresh PBMC	Control 1	NA	50	27	NA	5	0
	Control 2	NA	30	18	NA	17	2
	Control 3	68	43	41	54	3	5
	Control 4	25	27	11	9	5	3
	Control 5	40	41	8	39	55	31
NK cells from frozen PBMC and stored at 37^0 C overnight	Control 6	41	6	5	1	6	1
	Control 7	NA	20	35	NA	NA	NA
	Control 8	NA	33	34	NA	NA	NA

NA – data not available

8.3.2 Use of MHC class 1 negative EBV infected cell line as universal target

8.3.2.1 Characterization of 721.221 cell line

As the first method could not be reliably used for IM cases, a second method was optimized using a MHC class 1 negative EBV infected cell line as universal target for all controls and IM cases. 721.221 is a mutant EBV latently infected cell line which does not express MHC 1 (Shimizu et al., 1988), and is routinely used as a NK cell target. In order to ensure that the cell line was comparable to an autologous LCL, the expression of EBV latent antigens was monitored.

Flow cytometry experiments showed that the cell line was MHC 1 negative, but MHC 2 positive, and could therefore be used as a target cell for NK cell killing by any donor (Figure 8.9 page 155). Cytospins preparations from the 721.221 cell line were stained for the EBV latent antigens LMP, EBNA2 and EBNA 3a, 3b, in addition to CD19 (B cell antigen), and all were shown to be expressed by immunohistochemistry. The cell line was also positive for EBERS using In Situ hybridization. Immunohistochemistry using anti EBNA 3c antibodies failed, and antibodies to EBNA 1 were not available. These results confirmed a viral latency 3 pattern of gene expression in the 721.221 line (Figure 8.10 page 156).

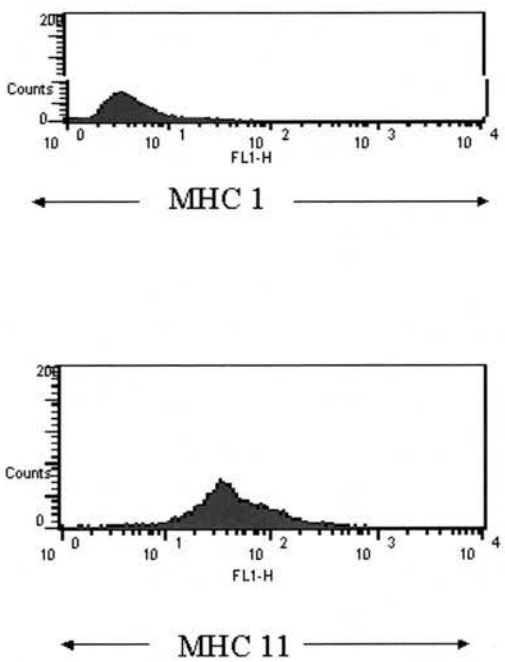
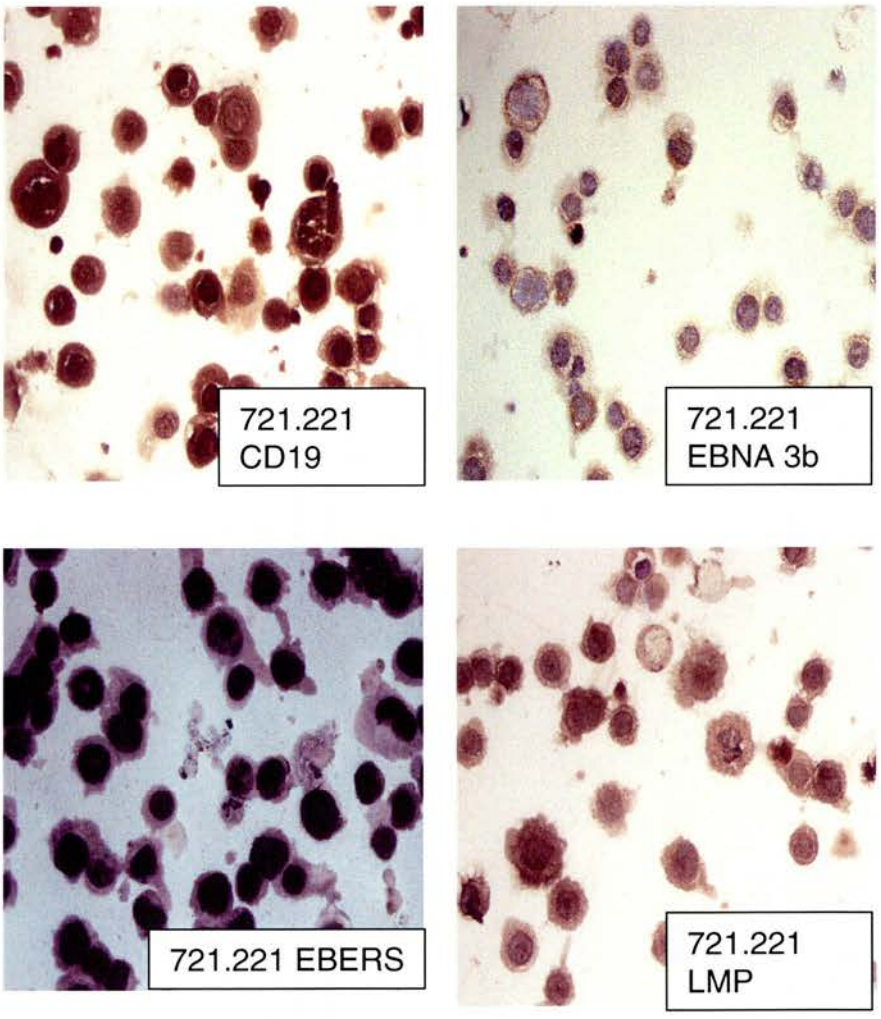


Figure 8-9 Cell line 721.221 does not express MHC 1.

The 721.221 cell line was stained with antibodies to both MHC1 and MHC2 and the expression of the antigens was assessed by flow cytometry. The histograms show negative expression of MHC1 (top histogram) and positive expression of MHC2 (bottom histogram).

Figure 8.10 shows cell line 721.221 stained with EBV latent antigens (stained brown) as indicated on each image. All images are magnified x 400



8.3.2.2 NK cells in IM show an enhanced capacity to kill an EBV infected cell line

NK cells were separated from 9 controls and 8 cases of IM and used as effector cells with the 721.221 cell line as the target, in a standard chromium release cytotoxicity assay. All NK cells were obtained from fresh PBMC, and used either directly or stored overnight at 4^0 C.

Fresh NK cells from acute IM showed an enhanced ability to lyse the 721.221 cell line compared to NK cells from controls, at ratios of 20:1 (median 8 % for controls and 32 % for cases), 10:1 (median 6% for controls and 20 % for cases) and 5:1 (median 5 % for controls and 14.5 % for cases). The differences in median percentage killing between controls and cases were statistically significant at both 20:1 and 5:1 ($p<0.05$), and just failed to reach significance at 10:1 ($p= 0.06$). A representative experiment is shown in Figure 8.11 page 157 and the results from controls (n=9) and IM cases (n=8) are shown in (Table 8-5 page 158).

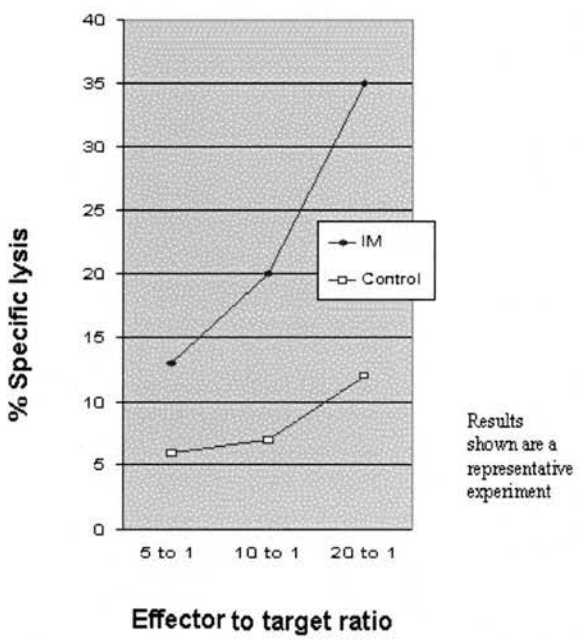


Figure 8-11 NK cells from IM patients have enhanced cytotoxic capacity against an EBV infected cell line

The percentage specific lysis of 721.221 target cells by NK effector cells at ratios of effector to target cells of 5:1,10:1 and 20:1 are plotted. A representative experiment from the results on the 8 IM and the 9 control experiments is shown.

Table 8-5 Percentage killing of K562 and 721.221 cell lines by NK cells

Acute IM	% Killing of target cells					
	Ratio of NK cell to K562			Ratio of NK cell to 721.221		
	20:1	10:1	5:1	20:1	10:1	5:1
Case 1	33	26	23	19	20	16
2	42	43	27	36	46	26
3	33	13	20	28	50	65
4	Na	16	10	Na	9	9
5	38	29	19	33	25	20
6	3	4	2	3	3	3
7	35	20	14	35	20	13
8	39	16	4	32	17	11
Range	3-42	4-43	2-27	3-36	3-50	9-65
Median	35	18	16.5	32*	20#	14.5*

*Median values were significantly different to control values ($p<0.05$)

Median values were of borderline difference to control values ($p=0.06$)

Healthy Ccntrols	% Killing of Target Cells					
	Ratio of NK cell to K562			Ratio of NK cell to 721.221		
	20:1	10:1	5:1	20:1	10:1	5:1
Control 1	19	14	8	12	7	6
2	21	16	11	19	11	8
3	Na	1	3	1	2	2
4	Na	Na	Na	4	3	5
5	Na	Na	Na	8	5	4
6	54	40	27	66	53	36
7	1	1	1	2	1	1
8	29	18	12	14	12	5
9	6	9	8	1	Na	Na
Range	1-54	1-40	1-27	1-66	1-53	1-36
Median	20	14	8	8	6	5

8.4 Percentage of NK cells in the peripheral blood is higher in those with mild compared to severe sore throat

The results of the functional studies on NK cells from IM cases suggest NK cells can eliminate EBV infected B cells, and therefore would have a protective role in primary EBV infection. In order to investigate this further we analysed levels of NK cells in peripheral blood in relation to clinical outcome.

As previously documented severity of sore throat was recorded in 26 students and classified as either mild (14 subjects) or severe (12 subjects) (Table 5-6 page 107). The percentage of NK cells in the PBMC was significantly higher in the mild compared to the severe group ($p<0.05$, Figure 8.12 page 159). The range of NK cells for the mild group was 8-38 %, with a median of 19.5%, and the range for severe group was 5-28 %, with a median of 13.5%. (The range for the control group was 1-19%, median 10%, see Figure 8.12 page 159). The number of NK cells was higher in the mild compared to the severe group – median of 0.87 x 10^6 /ml compared to 0.80 x 10^6 /ml respectively – however the difference was not significant.

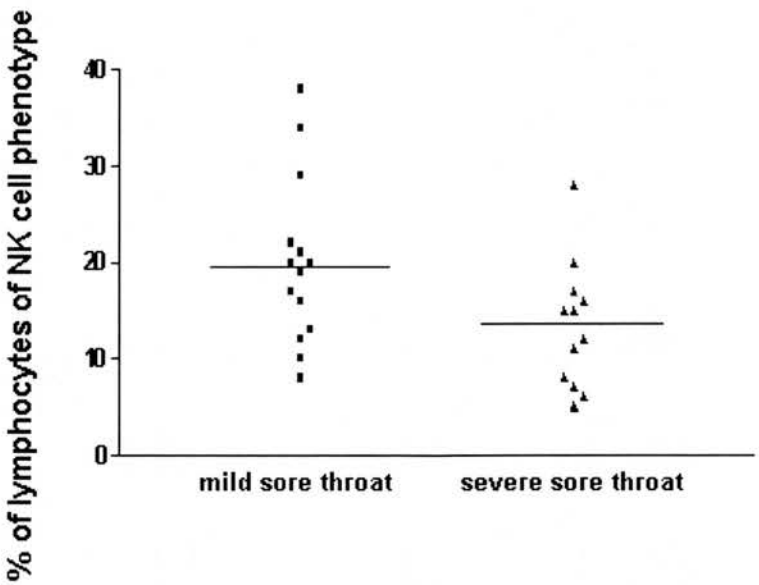


Figure 8-12 The percentage of NK cells in peripheral blood in acute IM is higher in those with mild compared to severe sore throat

A plot is shown of the percentage of lymphocytes of NK cell phenotype in the peripheral blood at diagnosis of IM for those with mild (N=14) (filled squares) compared to severe sore throat (N=12) (filled triangles). The bars show the median values of 19.5% for the mild sore throat group and 13.5% for the severe sore throat group ($p< 0.05$).

The relationship between a number of other clinical parameters and NK cells was also investigated. Fever was experienced by 21 out of 26 cases, and the median percentage of NK cells in the PBMC was higher in those without (20%) compared to those with fever (15%). However no significant statistical difference was found between the 2 groups, possibly because the group without fever was so small. Likewise, NK cell numbers in the peripheral blood were higher in those without fever (1.14 x 10^6/ml), compared to those with fever (0.87 x 10^6/ml), but again no significant statistical difference was found between the 2 groups. A rash occurred in only 5 of the 26 cases.

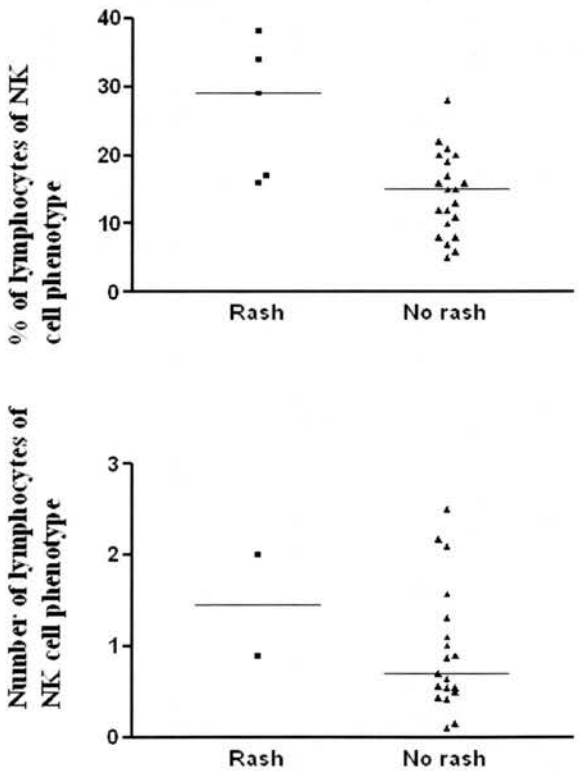


Figure 8-13 The percentage of NK cells in peripheral blood in acute IM is higher in those with rash than those with no rash

Plots are shown of both the percentage (upper plot) and numbers of lymphocytes (lower plot) of NK cell phenotype in the peripheral blood at diagnosis of IM for those with rash (N=5) (filled squares) compared to those with no rash (N=20) (filled triangles). The median percentage of NK cells was significantly higher at 29% in those with rash, compared to 15% in those without rash ($p<0.05$). The number of NK cells in the peripheral blood was also markedly higher in the group with rash, at 1.45 x 10^6/ml compared to 0.70 x 10^6/ml to those without rash, however changes were

not significant. FBC were not available in all cases. Median values are shown by the bars.

The median percentage of NK cells in the peripheral blood in the group with rash was significantly higher at 29% compared to 15% in those without rash ($p<0.05$, Figure 8.13 page 160). The number of NK cells in the peripheral blood was also markedly higher in the group with rash, at 1.45 x 10^6 /ml compared to 0.70 x 10^6 /ml to those without rash, however changes were not statistically significant, and there were only 2 cases in the group with rash.

Physical activity and fatigue were also recorded in the IM cohort. At diagnosis of IM, 17 of the 26 cases were able to leave home and 9 were house bound. Both the percentage and number of NK cells were lower in the housebound group (median values for the housebound group were 13% and 0.6 x 10^6 /ml, compared to 17% and 0.9 x 10^6 /ml for the group able to leave home). However, no significant statistical difference was found between the 2 groups. Assessment of ability to attend university was also made. 17 of the 26 cases either were not able to attend at all or attended part time, 6 cases continued to attend full time, and 3 cases were on holiday so were excluded from the analysis. No difference was found between the 2 groups either with respect to median number of NK cells or median percentage of NK cells in the peripheral blood. The median number of NK cells in the full time attendees was 0. 94 x 10^6 /ml compared to 0.90 x 10^6 /ml in the part time attendees, with median percentage of 17.5% and 16% respectively.

8.5 In acute IM, NK cells show an inverse correlation with EBV load

Next we investigated the relationship between EBV load and NK cell numbers at diagnosis of IM. Viral load was measured by PCR in the peripheral blood mononuclear cells of 23 IM cases. The range of viral loads was 0 to 49249 per 10^6 PBMC, and increased viral load correlated inversely with the percentage of NK cells in the peripheral blood, the pearson correlation coefficient ® was – 0.44 with corresponding p value of <0.05 (Figure 8.14 page 162). In a subgroup of 19 cases in whom lymphocyte counts were available, there was a trend to an inverse correlation

between numbers of lymphocytes in the peripheral blood and viral load but this was not statistically significant.

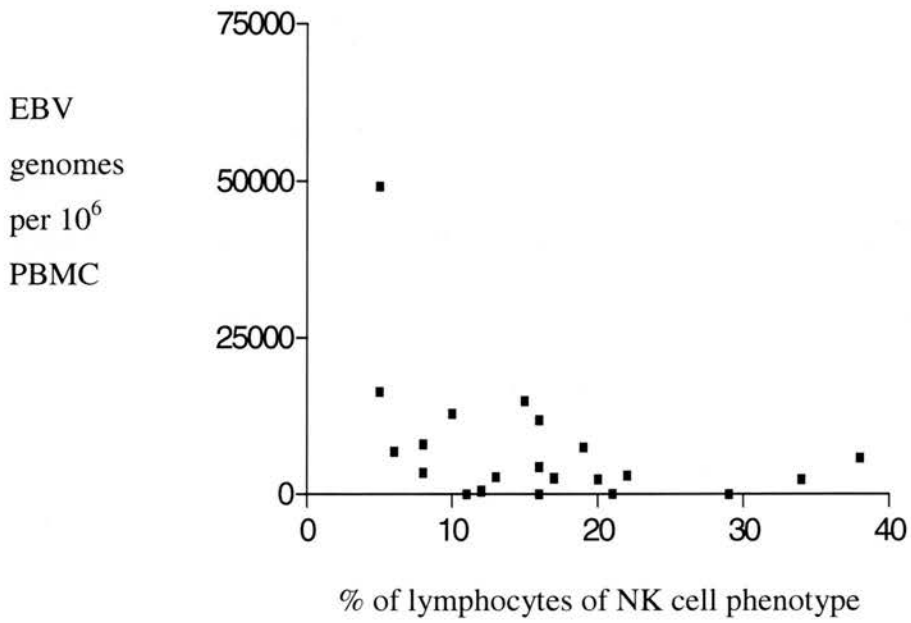


Figure 8-14 At diagnosis of IM the percentage of NK cells in the peripheral blood shows an inverse correlation with viral load

A plot is shown of EBV load per 10^6 PBMC against percentage of NK cells in the lymphocyte population at diagnosis of acute IM. The Pearson correlation coefficient ® was – 0.44 with corresponding p value of <0.05.

9 Discussion

Primary EBV infection presents a wide spectrum of clinical manifestations ranging from subclinical infection to fulminant disease, which is rapidly fatal (XLP). IM is caused by delayed primary infection with EBV and is thought to be immunopathological in nature, due to an exaggerated CD8+ T cell response to viral antigens presented on infected B lymphocytes. This CD8+ T cell response is now well characterised, and is antigen specific with large clonal expansions of cytotoxic T cells reactive with predominantly lytic viral antigens (see Section 2.6.8.1 page 34).

However the mechanisms underlying this intense immune activation and in particular its relationship to the clinical symptoms of IM, are not clearly understood. In addition it is not known why the majority of individuals undergo silent seroconversion, with IM limited to around fifty percent of those who seroconvert as young adults. A number of possible explanations have been proposed. These include variability of infecting dose, genetic diversity leading to alterations in the dynamics of the immune response such as cytokine polymorphisms, and factors in T cell development such as affinity maturation (Silins et al., 2001). Another factor may be changes in the immune response with age.

Recent progress in our understanding of fatal IM has identified a lymphocyte activation pathway in which cell surface CD244 and SLAM are regulated by SAP, the gene mutated in XLP. Since absence of a functional SAP protein in XLP leads to fatal IM, the level of control of immune activation by SAP is likely to be critical in determining the outcome of primary EBV infection in healthy individuals. A large, ongoing epidemiological study on IM in the Edinburgh student population (Crawford et al., 2002), allowed access to blood samples from subjects with acute IM and follow up samples on a subset of these for the present study.

9.1 Characteristics of the IM Cohort

Over 2 years we recruited 2012 first year students at admission to university to the MRC seroepidemiological study, and at this time around 25% were seronegative for EBV, both by serological and PCR testing. The seronegative individuals were asked to report to the university health service with clinical illness suggestive of IM. In total 137 cases of IM were diagnosed at the health centre over this period, and 56 cases were recruited, therefore we recruited 41% of all cases. The age range was 18-26, and around half the cases were male and half female. When compared to the epidemiological studies carried out in the 1960's, our cohort has a higher rate of sero prevalence to EBV. This is likely to be due to a more diverse social mix in university students and an increase in sexual activity in adolescence. Unlike the previous studies, our study included female students. Interestingly, we found female students had a higher seroprevalence of EBV at this age (Crawford et al., 2002), and therefore their inclusion would raise the overall prevalence of EBV in the cohort. The reason for this difference in seropositivity in men and women is unclear. One possibility is that the quantity of EBV a seronegative subject receives during sexual intercourse is higher from sperm, than from vaginal secretions, thus women, as the receptive sexual partner are more likely to seroconvert following intercourse with a seropositive partner. However, this hypothesis is only plausible if sexual transmission is an important mode of EBV spread. Alternatively, adolescent females frequently have sexual partners older than themselves, who in turn are more likely to be EBV positive as seropositivity increases with age.

The results of the clinical assessment have not been fully analysed, however our impression at this stage is that the clinical features have not changed markedly since the work on IM in the 1960's. The typical features continued to be sore throat experienced by over 95%, lymphadenopathy experienced by nearly 100%, and fever experienced by 75% of our cohort. Anorexia and headache were also common, experienced by around 78% and 66% respectively, and rash occurred in 10%. In addition the clinical course of the illness remains similar, with a short prodrome, of a median of 6 days followed by as severe period of a median of 7 days, followed by a prolonged recovery period. The median length of illness from the beginning of the prodrome was 32 days. Of interest, in the 1960's cases were often hospitalised for

several weeks (Sawyer et al., 1971), and though this does not occur now, a large number of cases suffered fatigue which led to significant impact on university studies. Unfortunately 4 out of the 56 cases studied either dropped down a year or left university following IM.

One of our aims was to make an objective assessment of disease severity at diagnosis. In order to do this we needed to identify clinical features that were common, varied with degree of illness, and could be recalled and recorded objectively. We found sore throat was the most useful parameter; it was both widely experienced and simple to grade. Furthermore we found sore throat was a reasonable surrogate marker of disease severity. For example experience of fever was easy to elucidate from the cases, but not a good marker for the spread of disease severity as it occurred in the vast majority of cases. However, the 5 cases that did not have fever had mild sore throat, confirming sore throat is a good surrogate marker of disease severity.

Analysis of physical function and fatigue was best assessed by ability to leave home; it is a simple fact to recall and does not require any further quantification. 9 out of the 26 cases were unable to leave home, and 1 of these cases had no fever and only mild sore throat whereas 2 of these cases had mild sore throat but fever. Thus there is a link between severity of clinical features and ability to leave home, but this was not absolute.

The high incidence of IM in the student population meant the GP's had a low thresh-hold for carrying out diagnostic serology. Consequently we identified a number of cases that had very mild illness. For example, nearly 20% (6 out of 26 cases) of the immune activation cohort managed to continue to attend university full time during their illness. One of the important conclusions from these data is that IM has a spectrum of illness from silent seroconversion, through mild illness to the more typical clinical course and finally those with severe IM.

Dr Macsween will report on the clinical aspects of the study in more detail in her MD thesis.

9.2 Immune activation study

A detailed analysis of the immune response and expression of the CD244/SLAM/SAP pathway in T cells was made in 26 cases of IM. In agreement with earlier studies discussed in the introduction (Section 2.6.8.1 page 34), we found a lymphocytosis in 85% of cases of acute IM at the time of diagnosis which mainly consisted of CD8+ T cells although NK cell numbers were also significantly elevated. In addition, several studies have documented the expression of T cell activation markers such as HLA DR and CD25 on CD8+ T cells in peripheral blood in IM (Tomkinson et al., 1987; Callan et al., 1996; Hoshino et al., 1999).

We have demonstrated that CD8+ T cells also show upregulation of the CD244/SLAM activation pathway. Thus, significantly increased numbers of CD8 T cells expressed cell surface CD244 and SLAM, and although absolute numbers of CD4 T cells were not raised, their level of activation as assessed by CD244 expression was also significantly increased. Earlier studies have shown that the activated T cells in IM secrete a variety of inflammatory cytokines (see Section 2.6.8.2 page 36), which are thought to be responsible for the clinical symptoms of IM. Our results now indicate that activation of CD4+ and CD8+ T cells through the CD244 and SLAM pathway is involved in this process. The change in activation status of CD4 + T cells in particular may be pivotal to directing the cytokine profile seen in IM by their ability to regulate the TH1/TH2 balance (Dong and Flavell, 2001).

Full recovery from IM is the norm, but the clinical course is often prolonged. Having found that the CD244/SLAM pathway is activated in IM T cells, we were interested to see whether this could be a key event in determining the disease pattern in IM by linking T cell activation with clinical events and duration of illness. Our results show that the prolonged clinical features are not simply a reflection of increased T cell numbers, as the lymphocytosis present at diagnosis fell rapidly, and by day 16 all T cell subsets were within the normal (control) range. However, the analysis also showed that, despite normal cell counts, T cells maintain expression of the activation markers CD244 and SLAM for prolonged periods following diagnosis. At around one month after diagnosis 5 out of 5 cases remained unwell, and in each

case expression of CD244 and SLAM on CD8 T cells was elevated. Moreover, those who had a more protracted clinical course continued to show increased expression of the activation markers on CD8 T cells even at 40 days post diagnosis. This work is in agreement with Bharadwaj et al who also identified T cells with an activated phenotype (CD38 positive) during recovery from IM (Bharadwaj et al., 2001).

Interestingly, even at an early stage of the disease process, we found that SAP expression was markedly increased in PBMC, thus maintaining control of the activation process. This contrasts with the situation in XLP where the loss of SAP function allows uncontrolled T cell activation and overproduction of inflammatory cytokines, usually with fatal consequences. Further evidence for the role of SAP in the pathogenesis of IM, was provided by the identification of the co-expression of SAP and INF γ in PBMC from acute IM.

As discussed, sore throat is a good marker of disease severity in IM. Symptoms vary from mild pharyngitis to a complete inability to swallow, and occasionally airway obstruction requires emergency surgical intervention. We graded the degree of sore throat at diagnosis as either mild or severe and found that those with severe sore throat had a significantly higher number of both CD8+ T cells, and CD8+ T cells expressing CD244. A small number of cases in our cohort did not experience fever, and again we found lower numbers of both CD8 and CD244/CD8 T cells in the group with less severe symptoms. In the same group of patients ability to leave home during acute IM had a weaker association with T cell activation, a finding that may reflect the pathology of fever and sore throat compared to the mechanisms underlying the fatigue affecting an individual's ability to leave home. Both fever and swelling with ulceration of the pharynx are a direct consequence of immune activation and cytokine release, whereas ability to leave home may reflect infection severity but also psychological response to illness.

Additionally some variables such as ability to attend university at the time of diagnosis were more difficult to quantify accurately and compare between individuals. We found students work load varied both between different courses and year of course. Therefore someone with the same degree of illness might manage to attend a small number of lectures in a week but not an all day field trip.

Of the immune parameters investigated numbers of CD8+T cells and CD244+CD8+ T cells had the strongest correlations with clinical features. SLAM positive subsets of T cells had a weaker link with clinical features and this suggests that CD244 subsets have a more significant role in the pathogenesis of IM. As CD3+ T cells are largely composed of CD8+ T cells in IM, the correlations found between CD3+T cell subsets and clinical features are explained by this CD8+ component.

We used a quantitative PCR method to measure the level of viral DNA in PBMC and over 90 % of cases had a detectable level of virus. These results are similar to Hopwood et al and Berger et al who detected EBV viral DNA in around 90% of IM cases studied (Hopwood et al., 2002). In addition Berger found levels of virus fell over time following diagnosis (Berger et al., 2001). We were interested in the relationship between viral load and clinical disease. We found that the percentages of both CD8+ and CD8+/CD244+ T cells correlated with the level of viral DNA in PBMC (viral load). Moreover, in 23 cases investigated the level of cell associated virus was higher in those with severe compared to mild sore throat (Figure 6-11 page 127). Therefore our data supports the hypothesis that higher viral loads are associated with increased immune activation and increased severity of outcome of infection, and by inference silent seroconversion would be linked to lower viral load. In contrast, in a small number of cases, Silins et al found similar levels of viral DNA in peripheral blood cells from those with silent and clinical seroconversion (Silins et al., 2001).

Since defective SAP function in XLP confers a specific inability to control EBV infection (Section 2.7.8 page 45), we were interested to find out whether activation of the CD244/SLAM/SAP pathway was in any way a specific feature of primary EBV infection. We therefore analysed expression of these molecules in PBMC from adults with primary VZV infection. We found that expression of CD244 was significantly enhanced in VZV cases compared with healthy controls, but that the changes were significantly more pronounced in IM despite the fact the VZV cases were more unwell with 3 out of 4 cases requiring hospital admission. (None of the 26 cases of IM in the immune activation cohort required hospital admission). One reason for this difference may be that VZV is directly cytopathic, with the virus itself causing cell damage and thus clinical features such as necrotic skin lesions (Arvin,

2003). Therefore in contrast to EBV the severity of clinical illness is not likely to be related to the degree of immune activation.

9.3 The NK cell response in IM

In contrast to the acquired immune response, little is known about innate immune responses during IM. Previous investigators have identified elevated NK cells and gamma delta T cell numbers, but information on function was hampered by difficulties in identifying a true NK cell population. However, identification of an NK cell defect in individuals with XLP highlights the importance of these cells in the control of primary infection (see section 2.10.6 page 61). Moreover, there is increasing evidence from experimental systems of a major role for NK cells in control of herpes virus infections (reviewed in Arase and Lanier, 2002). The best evidence comes from the demonstration of susceptibility to mouse CMV being linked to an activating NK cell receptor. At present, data from human infections are limited. We analysed the scale, phenotype and function of the NK cell response in the same cohort of 26 cases of IM.

We found a significant increase in NK cells at diagnosis of IM, with raised cell numbers and an elevated percentage of NK cells within the lymphocyte population. Furthermore, in a subgroup of patients from whom serial samples were available, we showed that median NK cell numbers remained elevated over the first month of the illness but had reverted to normal levels by 6 months post diagnosis. This finding contrasts with lymphocyte counts and CD8 + T cell numbers that rapidly returned to control levels. These data are in agreement with earlier studies showing elevations of CD16+ lymphocytes in IM (Tomkinson et al., 1987). We did not find an expansion of NKT cells in acute IM, however the characterisation of NKT cells remains difficult, and use of an alternative marker such as Vα14-Jα18/vβ11may be useful (Prussin and Foster, 1997; Lee et al., 2002).

In addition to the increase in NK cell numbers found in IM, we also identified a marked change in NK cell phenotype, including a significant increase in the proportion of $CD56^{bright}$ cells in the peripheral blood. It should be noted that, as the total numbers of NK cells are raised there were increased numbers of both $CD56^{bright}$

and $CD56^{dim}$ subsets, but with an expansion of the proportion of the $CD56^{bright}$ subset in cases compared to controls. These $CD56^{bright}$ cells are potent producers of immunoregulatory cytokines, and recent studies by Fehniger et al, identified them as the dominant NK cell subset in human lymph nodes. They show that $CD56^{bright}$ cells release cytokines such as INF γ which can stimulate antigen specific T cells (Fehniger et al., 2002). Activated T cells then release IL-2, which in turn activates $CD56^{bright}$ cells to become highly cytotoxic and also stimulates the production of more INF γ. This stimulatory loop thus provides an important link between the innate and adaptive immune responses. In the context of primary EBV infection, where initial infection of B cells occurs in the lymphoid tissue of the oropharnyx (mainly in tonsil), the level of interaction between activated $CD56^{bright}$ cells and EBV specific T cells may be key to determining clinical outcome. It is possible that the increased numbers of $CD56^{bright}$ cells which we detected in peripheral blood represent a spill-over from the primary site of infection, but clearly it would be interesting to study NK and T cell subsets, along with cytokine gene expression, in tonsils from early EBV infections. The co-expression of CD16 on $CD56^{bright}$ NK cells has not been previously described, and its significance is unclear.

The functional studies revealed that freshly isolated NK cells from IM blood show an enhanced ability to kill an EBV infected B cell line, and we suggest that this is a key factor in control of the early infection. In primary infection EBV establishes a pool of infected B cells in oropharyngeal lymphoid tissue both by driving their proliferation and by inducing the production of viral progeny, which can infect bystanding B cells. Infected B cells from this site enter the blood stream and disseminate throughout the body. In healthy individuals the immune system eventually exerts control over this infection and a state of viral latency is established. EBV specific cytotoxic T cells are believed to be key to the resolution of primary infection. However, these cells appear to develop slowly since there is an incubation period of around 30 days before the typical immunopathological symptoms of IM develop. Thus there is a window of opportunity for uncontrolled B cell proliferation and viral replication, and it is during this period that the cytotoxic activity of NK cells is likely to be crucial. Moreover, Parham (Parham, 2003) has recently suggested that early control of infection by the innate immune system, and NK cells in

particular, may result in resolution of infection prior to symptom onset. This argument is especially relevant to primary EBV infection where the pathology of symptomatic infection, IM is driven by the acquired immune response to the virus rather than the virus directly. Another interesting possibility is that NK cell phenotype and function is related to age and cell function in infants may allow better control of early viral infection.

The importance of NK cells in control of primary infection is supported by our analysis of disease severity in relation to NK cell responses. In contrast to CD8+ T cell responses, where higher numbers of cells were linked to more severe illness; higher percentages of NK cells were found in the peripheral blood of those with milder illness. Additionally, we found an inverse correlation between percentage of NK cells and amount of EBV load in the peripheral blood (Section 8.5 page 161). However, we have also found a positive correlation between the percentage of CD8+T cells in the peripheral blood and viral load. Therefore it is not clear whether both CD8+T cell and NK cell responses independently influence viral production, or whether CD8+ T cells are the driving force and lower percentages of NK cells in the peripheral blood are simply a reflection of higher percentages of CD8+T cells. Our results are only an indication of the relationship between the factors at the point of diagnosis, and it would be useful to monitor the 3 factors in a larger number of cases over the course of the illness. Interestingly in the 6 cases in which we monitored NK cell counts over time, those with more severe illness as assessed by sore throat severity all had lower NK cell counts than those with mild sore throats (Figure 8-4 page 145).

There was not a clearcut relationship between NK cell counts and physical function such as ability to attend university. This may reflect that NK cell function is most critical in early stages of disease pathology, and so links with the first physical symptoms such as sore throat, and not with later and prolonged features such as impaired physical function. In summary we have provided evidence that NK cells are a critical component of the control of primary EBV infection in adults with IM.

Interestingly, our work has parallels in a mouse model of CMV infection, where increased NK cell cytotoxicity has been identified very early in viral infection, and was mediated by virus induced IFN α and IFN β (Orange and Biron, 1996).

9.4 XLP mutation analysis

We screened 10 cases of putative XLP for mutations in the four coding exons of the SAP gene, using PCR based sequencing techniques, and found no mutations. The most likely explanation for this is that the cases are in fact sporadic fulminant IM. The cases were all males who had been suspected on clinical grounds of having severe EBV related disease, in the majority of cases presenting with severe fatal IM. Clinical data was very limited in a number of cases, with only 2 of the cases having a family history, and in several of the cases the evidence to suggest a diagnosis of XLP was fairly limited. However, at the time of identification of the XLP gene, it was important to establish whether mutations in SAP were linked to other conditions in which severe EBV infection occurs. In other reported series 50-60% of those with a clinical diagnosis of XLP have been found to have a mutation. Another explanation is that mutations may be present in parts of the gene that have not been sequenced such as the promotor regions. This work reinforces the importance of establishing good diagnostic criteria for a condition before attempting to find putative genes linked to disease causation.

Since the identification of the XLP gene, a great deal of progress has been made in our understanding of the pathogenesis of the disorder, and the critical role of SAP in control of lymphocyte activation. It is now clear that clinical XLP can occur without EBV infection, but the virus continues to be the most well recognised trigger. Why this should be so is still not known. One hypothesis is that NK cells are vital to the control of primary EBV. Thus the loss of control of NK cell activation due to mutations in SAP in XLP patients, may lead to a failure of appropriate NK cell killing via an interaction of CD244 on the NK cell and CD48 on EBV infected B cells. However, NK cells appear to be important to the control of other viral infections such as CMV, and CMV is not a common trigger for XLP.

Another intriguing possibility is suggested by recent work from a mouse model of XLP. Mice with mutations in the SAP/SH2D1A gene were found to have an inability to develop full humoral immunity following viral infection, as their B cells failed to switch immunoglobulin isotype (Crotty et al., 2003). Interestingly, this was thought to be due to lack of appropriate CD4+T cell help, rather than an overt defect in B cells, as SAP is predominately expressed in T cells rather than B cells. This failure to switch immunoglobulin isotype would explain the hypogammaglobulinamia which is a characteristic feature of XLP. Critically, resolution of primary EBV infection and change to latent infection requires a switch in B cell phenotype from a proliferating B cell blast into a B memory cell. This switch to a memory cell phenotype is associated with a down regulation in the immune response, as the immune stimulating EBV antigens presented on the proliferating blast are down regulated. Thus if the mouse model reflects what occurs in human XLP patients, EBV infected B cells would be unable to undergo this change in phenotype. Interestingly this is probably not directly due to a defect in the B cells as SAP does not appear to be expressed in B cells, but due to defective CD4+ T cell help. Therefore the EBV infected blast, cannot switch to a memory phenotype and so provides a continuous immune stimulus for lymphocyte proliferation that is characteristic of XLP.

It has been suggested that SAP expression may differ between individuals or with age (Klein and Klein, 1998), and this could explain the age related variation in response to primary EBV infection. The work we have carried out so far is not extensive enough to clarify this, although we did notice considerable variation in the numbers of PBMC expressing SAP between healthy donors. It would certainly be interesting to investigate SAP expression and immune activation pathways in infants, but there are considerable ethical difficulties in carrying out studies in healthy children.

However we are looking for polymorphisms in the SAP gene, which might underlie the variation in SAP expression which we have documented, and which in turn might explain the variable clinical response to primary infection in adults. Another possibility that might underlie a genetic susceptibility to clinical primary EBV infection is differences in the NK cell KIR family of receptors.

The KIR family of NK cell receptors is known to be one of the most polymorphic regions of the human genome, and several investigators have suggested that this genetic diversity is driven by common viral infections (Arase and Lanier, 2002). At present it is not clear whether certain KIR haplotypes are linked to disease susceptibility; but it would certainly be interesting to investigate this possibility in relation to clinical outcome of primary EBV infection.

Some of the difficulties we have in understanding the pathogenesis of IM would be helped by an animal model of IM, such as the rhesus monkey model developed by Wang (Wang, 2001). In humans we can compare immunological and virological characteristics between individuals with different clinical responses to infection, and from this build up an understanding of the relationship between them, as we have done in this study. However, there are limitations to this approach, which can establish correlations, but further information would be gained in a system which allowed controlled manipulation of the factors. For example it would be useful to quantify the changes in virus specific CD8 cells that occurred over the clinical course of the illness in animals infected with varied doses of virus. Another useful study would be to monitor the course of disease in animals depleted of NK cells.

The role of CD4+T cells in the control of primary EBV infection has not yet been fully elucidated. Therefore another useful addition to the study would be to monitor CD4+T cells, ideally EBV antigen specific T cells, and relate these findings to clinical features and viral load. Gamadia and colleagues have recently identified the early development of antigen specific CD4+ T cell responses as a key difference in the immune response in those that undergo silent as opposed to clinically overt CMV infection post transplant (Gamadia et al., 2003). Parallels may occur in primary EBV infection and it would be interesting to investigate whether mild primary EBV infection is associated with increased number of virus specific CD4 + T cells.

We suggest that activated T cells expressing CD244 modulate the clinical features of IM, but control of activation is maintained by concurrent increased expression of SAP. However, before this occurs NK cells have a critical role in both eliminating infected B cells and augmenting this antigen specific T cell response via release of immunomodulatory cytokines. The magnitude of the NK cell response may

ultimately determine whether primary EBV infection has a subclinical or clinical outcome.

10 Future perspectives

It is clear from the data presented in this thesis that the scale of the acquired immune system is critical to the clinical outcome of primary EBV infection. Additionally, the data supports a complementary role for NK cells in control of EBV infected cells and possibly a link between the 2 components of the immune system. A number of questions are raised by this data.

Is there a functional difference between $CD56^{bright}$ cells and $CD56^{dim}$ cells in acute IM?

Flow cytometric sorting of $CD56^{bright}$ cells and $CD56^{dim}$ cells in acute IM and controls, followed either by RT-PCR studies or immunohistochemical staining of cytospins, would allow assessment of the cytokine profile of each cell type. In addition, information on serum levels of cytokines, both those involved in NK cell activation (such as type 1 interferons) or released from NK cells (INF γ), would clarify the cytokine networks important in NK cell control of EBV.

What is the molecular interaction between NK cells and EBV infected cells?

At present we know little of the molecular details of the interaction between NK cells and EBV infected cells. We do know that CD244 and CD48 interact in vitro; little is known of the functional importance of this in vivo. This could be investigated further by assessing NK cell mediated cytotoxicity of a cell line 1106MEL (Porgador et al., 1997) transfected (using vaccinia constructs) with individual EBV lytic and latent genes. Alternatively, monoclonal antibody blocking studies could be carried out using the model of NK mediated killing of an EBV infected cell line we have developed (Section 8.3.2.2 page 157). Antibodies to EBV lytic and latent antigens, as well as antibodies to NK cell surface receptors could be added to the system, and any inhibition of killing monitored.

Do NK cells have a role in the prevention/treatment of EBV associated tumours?

This could be investigated further in the (SCID) mouse model of BLPD by monitoring tumour response in mice treated with autologous human NK cell

infusions. Alternatively, NK cell numbers, function and phenotype could be monitored in transplant recipients and in parallel their EBV DNA load in the peripheral blood and incidence of BLPD development.

Does NK KIR receptor usage vary between silent and overt seroconvertors to EBV?

At the end of the MRC study DNA will be available from cases (over 50 in each group) that have silently seroconverted, cases who have developed IM and persistently seronegative individuals. It would be useful to investigate differences in KIR usage between these different groups, and this could be carried out using the PCR-SSP method recently developed by Uhrberg et al (Uhrberg et al., 1997; Gomez-Lozano and Vilches, 2002).

11 Reference

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Visit Number	Number MRC/	Date of Visit
Date of Birth	Doctor	

Clinical assessment - Visit 1

Date of first symptoms (incl "prodrome")		
Date of most severe symptoms		
Duration of most severe symptoms (days)		
Unwell for how long (days)		
Presenting features and how long experienced for at time of presentation to GP (choice of physical features below)	1	
	2	
	3	

Activities

		Date stopped	Date Returned
Are you able to attend all your timetabled classes	Yes/No		
Total hours timetabled per week			
Please quantify since onset of symptoms: Exams missed Tutorials Lectures Practicals Total hours missed due to illness			
Is the amount of studying that you do in your own time normal for you at present?	Yes/No		
Are you able to undertake your normal social life?	Yes/No		
Are you able to undertake your normal exercise pattern ?	Yes/No		
Do you have a part-time job?	Yes/No		
Hours per week			
If yes, give dates absent, or "none" for no time off			

Physical features

Sore throat	Have you experienced this ever during your illness	No, not at any point during illness
Unable to swallow saliva	YES/NO	
Unable to swallow liquids	YES/NO	
Unable to swallow soft diet	YES/NO	
Able to swallow but painful	YES/NO	
Sore throat	YES/NO	
Rash a) faint morbilliform b) antibiotic related, (which and timing) c) other	YES/NO	
Glands	YES/NO	
Fever	YES/NO	
Sweats	YES/NO	
Anorexia	YES/NO	
Headaches	YES/NO	
Muscle/joint aches	YES/NO	
Syncope Abdominal pain Other		NO
Do you have any other medical conditions, the glandular fever has affected?		
Alcohol - units in last week		
Alcohol – usual per week		

Admitted to hospital / seen in A+E / out-patients?

Visit Number	**Number MRC/**	**Date of Visit**

Examination

Pulse	
Blood pressure	

Temperature	*student will be given chart and thermometer to do daily checks, data recorded separately*			
Throat exudate	*YES/NO*			
Periorbital swelling	YES/NO			
Palatial petechiae	YES/NO			
Jaundice	YES/NO			
Rash	YES/NO			
Spleen size	Not palpable	Tip	Fingers below costal margin	
Liver	Not palpable	Edge	Fingers below costal margin	
Palpable glands	YES/NO	cervical	Right left both	
		supraclavicular	Right left both	
		Submandibular	Right left both	
		axilliary	Right left both	
		inguinal	Right left both	
Tonsils enlarged	YES /NO	Grade 1 2 3		

Visit Number	**Number MRC/**	**Date of Visit**
Date of Birth	**Doctor**	